Investigation into the *Saccharomyces cerevisiae* U5 snRNP, a core spliceosome component

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List of Abbreviations

A	Adenine
AMV	Avian Myeloblastosis Virus
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
C	Cytosine
Ci	Curie
Da	Daltons
d	Deoxy
dd	Dideoxy
DNA	Deoxyribonucleic acid
dpm	disintegrations per minute
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESMA	Electrophoretic mobility shift assay
FOA	Fluoroorotic acid
g	Gravitational force
G	Guanine
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid
His	Histidine
HSQC	Heteronuclear single quantum coherence
IGEPAL	Octylphenoxypolyethoxyethanol
IgG	Immunoglobulin G
IL	Internal loop
ISL	Internal stem loop
Ins	Insert
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
l	litre
LB	Luria-Bertani

m	milli
mg	milligrams
M	molar
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
NTP	Nucleoside triphosphate
OD	Optical density
p	pico
PCA	Phenol−chloroform−isoamyl alcohol
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PNK	Polynucleotide kinase
ppm	Parts per million
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcriptase
RT	Room temperature
SD	Synthetically defined
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonuclearprotein
Sub	Substitution
SOC	Super Optimal Broth
SSC	Sodium citrate/sodium chloride
T	Thymine
TAP	Tandem affinity purification
TBE	Tris/borate/EDTA
TE	Tris/EDTA
TOCSY	Total correlation spectroscopy

Tris	Tris(hydroxymethyl)methylamine
tRNA	Transfer RNA
U	Uracil
V	Volts
VSL	Variable stem loop
W	Watts
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XTP	Xanthosine 5'-triphosphate
YPD	Yeast-extract Peptone Dextrose
°C	Degrees Celsius
µ	micro

Amino acids

A	Alanine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
N	Asparagine
P	Proline
R	Arginine
S	Serine
T	Tyrosine

Abstract

The U5 snRNP is a major component of the yeast spliceosome, being part of the U4/U6.U5 tri-snRNP, the precatalytic spliceosome and the catalytically activated spliceosome. The U5 snRNP includes, at its heart, the U5 snRNA which contains the invariant Loop 1 that functions in tethering and aligning exons during splicing. The major protein components of the U5 snRNP are the highly conserved Prp8p, the GTPase Snu114p and the helicase Brr2p. These proteins and the U5 snRNA are integral in forming the active site of the spliceosome and regulating the dynamic changes of the spliceosome.

The first part of this study aimed to express and purify specific domains of Snu114p to define the structure and function of Snu114p. The N-terminal region of Snu114p was successfully expressed and purified from bacteria. Addition of the Snu114p N-terminal fragment to *in vitro* splicing assays resulted in a first step splicing defect, indicating a role for the N-terminus in pre-mRNA splicing. NMR studies revealed that the N-terminus of Snu114p exists as an unstructured protein domain. Mutagenesis indicated that the N-terminus of Snu114p is tolerant to mutation. A novel genetic interaction between amino acids in the N-terminus of Snu114p and the 3' side of the U5 snRNA IL1 was identified. It is proposed here that the N-terminus of Snu114p functions to stabilise interactions of Snu114p with other proteins or snRNAs, possibly the U5 snRNA. Alternatively, the N-terminus of Snu114p may form intramolecular interactions with other regions of Snu114p to regulate Snu114p function in pre-mRNA splicing.

Prp8p, Snu114p and Brr2p are known to form a stable complex but their interactions with the specific domains of the U5 snRNA are not known. The second part of this study aimed to investigate the association of Brr2p, Snu114p and Prp8p with the U5 snRNA. Mutants of the U5 snRNA were constructed in the conserved Loop 1 and the Internal Loop 1 (IL1). The influences of the U5 snRNA mutations on interactions of Prp8p, Snu114p or Brr2p with the snRNA were investigated. It was revealed that Loop 1 and both sides of IL1 of the U5 snRNA are important in association of Brr2p, Snu114p and Prp8p. Mutations in the 3' side of IL1 drastically reduce association of Brr2p, Snu114p and Prp8p with the U5 snRNA, highlighting this region as a potential 'protein docking' site within the U5 snRNP. Differences seen in the associations of Brr2p, Snu114p and Prp8p with U5 snRNA mutations demonstrate that although there are intimate interactions between Brr2p, Snu114p and Prp8p, they do not associate with the U5 snRNA as a tri-protein complex. Genetic screening of *BRR2* and U5 snRNA mutants reveals an interaction between the N-terminal half of Brr2p and the 3' side of U5 snRNA IL1. This supports the proposed 'protein docking' site at the 3' side of the U5 snRNA IL1.

Data presented in this study increases our understanding of the regions in the U5 snRNA required for association of the essential U5 snRNP proteins, Brr2p, Snu114p and Prp8p, and goes some way to elucidating the organisation of essential proteins within the U5 snRNP.

Declaration

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Dedication

This thesis is dedicated to my family. Mum, Dad, Sam and all of my Grandparents.

Thank you for all the love and support you have given me for all these years. Thank you for making me believe that I could get through my PhD and for keeping me positive and happy. I love you all!

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1 Introduction

Protein production is an essential process for all living organisms. The deoxyribonucleic acid (DNA) of a cell consists of non-coding and coding regions, with the coding regions containing information essential for production of all proteins molecules required by the cell. The first stage in protein production is transcription, during which RNA polymerase II transcribes protein-coding genes, resulting in the production of an RNA transcript, known as the precursor-messenger RNA (pre-mRNA). The pre-mRNA undergoes several modifications to convert it into mature messenger RNA (mRNA). Following successful modification and maturation of the pre-mRNA into mRNA, coding mRNA is exported from the nucleus into the cytoplasm to be used as a template for protein synthesis during the process of translation (Proudfoot et al., 2002).

1.1 The pre-mRNA modifications

To enable the transition of pre-mRNA into mRNA, a series of modifications have to take place. The first of these modifications is the addition of a 5' cap. The cap is added to the 5' end of mRNAs in a three-step reaction, after the synthesis of around 20 to 30 nucleotides of pre-mRNA. The three steps are carried out by three enzymes; an RNA 5' triphosphatase, a guanylyltransferase and a methyltransferase. The cap structure is recognised by the cap binding complex (CBC) and helps to protect the mRNA against degradation by hydrolytic enzymes. Upon export from the nucleus the CBC is replaced by the translation initiation factor eIF-4E (Proudfoot et al., 2002).

The coding DNA sequences within a gene are present in regions termed exons. These exons are separated by intervening sequences termed introns. Both introns and exons are transcribed. However, as introns interrupt the coding exons, they need to be removed prior to

translation, to achieve a translatable, functional mRNA. This process of intron removal was discovered in 1977 when branched structures were identified when carrying out electron microscopy of hybridised RNA-DNA molecules (Berget et al., 1977; Chow et al., 1977). It was observed that regions of non-hybridised RNA were actually complementary to regions of DNA upstream, rather than adjacent to the region that had hybridised. This led to the theory that there were non-complementary regions of DNA that were not part of the genes mRNA product and had to be removed (Berget et al., 1977; Chow et al., 1977). Intron removal is achieved through a process called pre-mRNA splicing. Pre-mRNA splicing involves the removal of introns from the pre-mRNA and ligation of the flanking exons, giving an uninterrupted coding sequence suitable for use as a template for protein production in translation.

The final modification of the pre-mRNA is the addition of adenosine residues to the 3' end of the pre-mRNA, a process called polyadenylation. Around 200 adenosines are added to mammalian pre-mRNA and around 75 are added to yeast pre-mRNAs (Manley, 1995). This is known as a poly (A) tail and its formation is dependent upon sequences present in the pre-mRNA and the polyadenylation machinery. Prior to addition of the poly (A) tail, the pre-mRNA is cleaved. In humans cleavage usually occurs at a CA dinucleotide, between a highly conserved AAUAAA hexamer, and a U or GU rich motif known as a downstream sequence element (DSE) (Proudfoot et al., 2002). In yeast cells the conserved hexamer is dispensable, often being absent (Manley, 1995). In humans polyadenylation involves the cleavage and polyadenylation stimulation factor (CPSF) and the cleavage factors 1 and 2 (CF1 and CF2). In yeast polyadenylation involves several multi subunit factors, namely cleavage factors 1A and 1B (CF1A and CF1B) and a cleavage and polyadenylation factor (CPF) (Proudfoot and O'Sullivan, 2002).

Successfully modified mRNA is exported from the nucleus via the nuclear pore complex (NPC), into the cytoplasm where ribosomes use it as a template for protein synthesis (Cole and Scarcelli, 2006; Proudfoot et al., 2002). Nuclear export of the bulk of spliced pre-mRNAs requires the transcript to be associated with a protein complex known as the THO complex, so called after its first known component Tho2p (Chavez et al., 2000). This occurs during transcription. Following pre-mRNA splicing a group of proteins known as the exon junction complex (EJC) are bound to the site of exon ligation. This EJC and the CBC are important for the association of the transcription-export complex (TREX). The TREX consists of the THO complex and several other proteins, including the DEAD-box protein Sub2p and the export factor Yra1p. Sub2p mediates interactions with the THO complex and Yra1p in an ATP dependent manner (Dufu et al., 2010). A heterodimer Mex67p/Mtr2p binds to the mRNA and facilitates docking to the NPC via interactions with NPC proteins known as FG-Nups (Carmody and Wente, 2009; Walsh et al., 2010). As transcripts enter the cytoplasm they are bound by Dbp5p and Gle1p, which triggers release of Mex67p/Mtr2p. Binding of the CBC is replaced by binding of the translation initiation factor eIF-4E. Gle1p also has a role in translation initiation and both Gle1p and Dbp5p are involved in translation termination (Carmody and Wente, 2009).

1.2 The importance of pre-mRNA splicing

Pre-mRNA splicing is a crucial event in the maturation of pre-mRNA and is essential for proper protein production in eukaryotic cells. Without correct and successful splicing, resulting proteins may well be mutant or non-functional, if translation can happen at all. However, this process is not as simple as it appears. Many pre-mRNAs contain multiple introns and exons, therefore have more than one splicing pattern, with some exons being

included in certain cell types or under certain conditions, and being spliced out in other cell types or under other conditions. This is known as alternative splicing and enables a single gene to encode several protein isoforms with diverse functions (Sharp, 1994). Many genes in yeast contain only a single intron, but genes in many other organisms do contain several introns and exons and produce several different proteins via alternative splicing. An extreme example of this is the Drosophila Dscam gene which contains 95 variable exons and can potentially produce 38016 different splicing isoforms from a single gene (Graveley, 2005; Smith, 2005).

There are also diseases caused by mutations that result in splicing defects. These mutations can be within RNA sequence motifs required for pre-mRNA splicing (these RNA sequences will be discussed later) and mutations in proteins involved in pre-mRNA splicing (Faustino and Cooper, 2003; Tazi et al., 2009). Diseases known to be linked to splicing defects include Frasier syndrome, Spinal muscular astrophy and the eye disease Retinitis Pigmentosa (Barbaux et al., 1997; Blencowe, 2000; Tazi et al., 2009). Increasing our understanding of the mechanisms behind pre-mRNA splicing and the splicing machinery, improves the prospects of understanding, treating and curing the numerous diseases and syndromes caused by defects in pre-mRNA splicing.

1.3 The conserved sequence elements of the pre-mRNA required for splicing

Specific RNA elements are required for introns to be recognised and pre-mRNA splicing to occur (Figure 1.1). In *S.cerevisiae* these are a 5' splice site (GUAUGU), a variable length of pyrimidines known as a polypyrimidine tract, a branch point region (UACUAAC) and a 3' splice site (YAG where Y represents a pyrimidine C or U) (Langford and Gallwitz, 1983; Langford et al., 1984; Spingola et al., 1999). Mutation of either of the GU nucleotides at the 5'

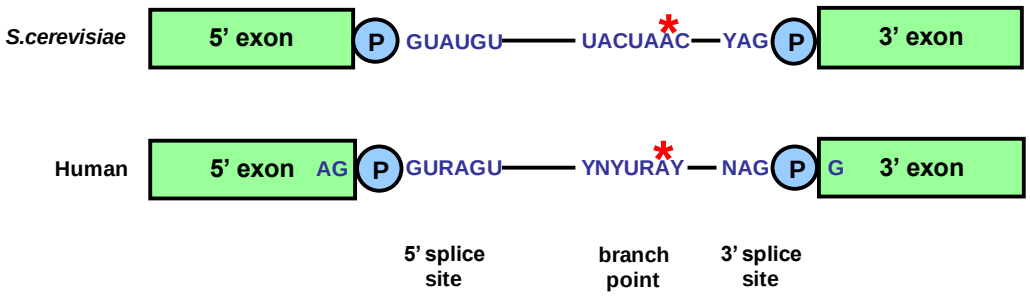


Figure 1.1 Comparison of *S.cerevisiae* and human pre-mRNA conserved sequence elements involved in pre-mRNA splicing. This figure illustrates the typical conserved sequences at *S.cerevisiae* and human 5' splice site, branch point and 3' splice site. Y represents a pyrimidine (C or U), R represents a purine (A or G) and N represents any nucleotide. The branch point adenosine is marked by a red asterisk. P represents the phosphates involved in the two transesterification steps.

splice site results in inactivation of the site (Montell et al., 1982; Parker and Guthrie, 1985; Treisman et al., 1982; Vijayraghavan et al., 1986). The GU dinucleotide at the 5' end of the intron is present in almost all naturally occurring eukaryotic introns. In human pre-mRNA these sequence elements are not as highly conserved (Figure 1.1). In a typical human pre-mRNA, the 5' and 3' splice sites consists of the sequence AG/GURAGU and NAG/G respectively, where N represents any of the four bases and R represents a purine (A or G) (Mount, 1982). The branch point region in human introns is not as highly conserved as it is *S.cerevisiae*. The human branch point consists of the loose consensus sequence, YNYURAY, where N represents any of the four bases, R represents a purine (A or G), and Y represents a pyrimidine (C or U), and is located between 18 and 37 nucleotides upstream of the 3' splice site (Reed and Maniatis, 1985; Spingola et al., 1999). These sequences can differ and are not present in all human pre-mRNAs. Mutation of the branch point region in humans leads to activation of cryptic branch points, whereas in yeast deletion of the branch point sequence prevents splicing (Ruskin et al., 1985; Vijayraghavan et al., 1986). The human and *S.cerevisiae* conserved splicing sequences are compared in Figure 1.1. In human pre-mRNA and other higher eukaryotes additional regulatory elements can also be present. These regulatory elements include intronic and exonic splicing enhancers (ISE and ESE, respectively) which promote splicing, and intronic and exonic splicing silencers (ISS and ESS, respectively) which silence splicing (Chasin, 2007).

1.4 The two steps of pre-mRNA splicing

Pre-mRNA splicing occurs via a two step transesterification reaction (Figure 1.2). The first catalytic step is cleavage at the 5' splice site, and involves nucleophilic attack of a phosphate

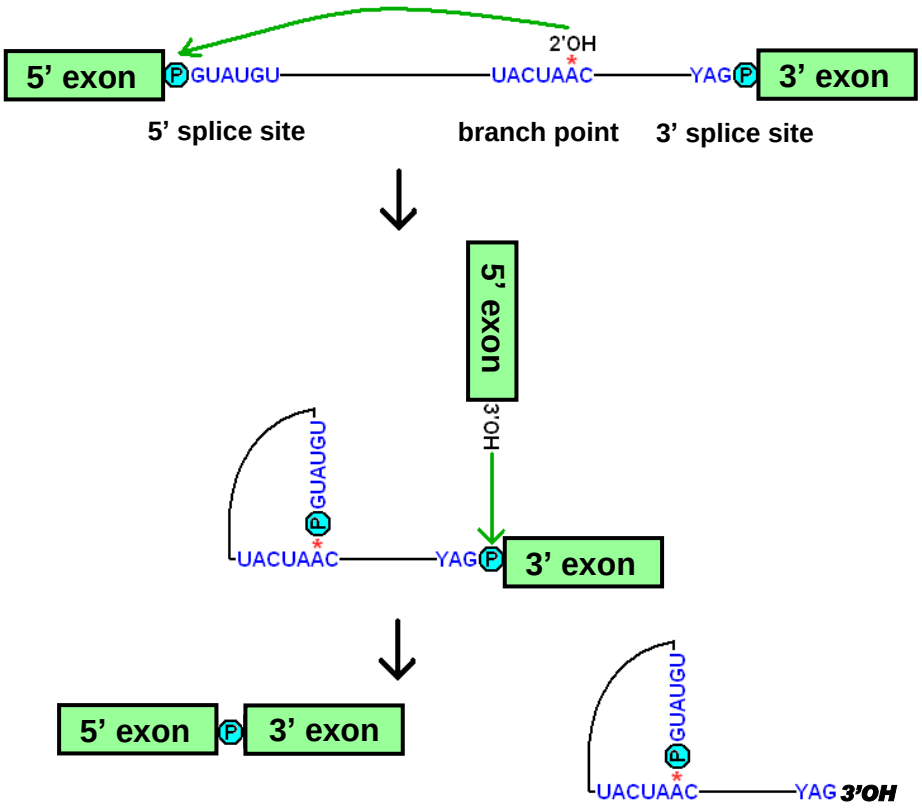


Figure 1.2 The two transesterification steps of pre-mRNA splicing. Intron removal occurs via a two-step transesterification reaction, resulting in the release of the intron in a lariat structure. The first step involves cleavage at the 5' splice site and formation of a branched molecule known as the lariat intermediate. The second step involves cleavage at the 3' splice site and ligation of the 5' and 3' exons. The position of the phosphate groups and OH groups involved in the reaction are illustrated above. The branch point adenosine is marked with a red asterisk.

at the 5' splice site by a 2' hydroxyl of an internal adenosine in the branch point region (Query et al., 1994). Due to the nature of base pairing between the branch point and the U2 snRNA, the branch point adenosine is bulged out of the RNA-RNA duplex (Query et al., 1994). This leads to cleavage of a phosphodiester bond at the 5' splice site and the formation of a 2'-5' phosphodiester bond between the 5' end of the intron and the branch point. This releases the 5' exon and forms a branched molecule, known as a lariat intermediate (Konarska et al., 1985). The second catalytic step is cleavage at the 3' splice site. This involves nucleophilic attack of the phosphate at the 3' splice site, by the 3' hydroxyl (OH) of the 5' exon. This results in release of the intron as a lariat and ligation of the two exons (Padgett et al., 1984). Both catalytic steps are activated via a two-metal-ion strategy, in which the 2' or 3' hydroxyl groups are activated for attack by metal ions (Gordon et al., 2000; Sontheimer et al., 1997). These two steps are driven by a large protein-RNA complex known as the spliceosome.

1.5 The spliceosome

During pre-mRNA splicing introns are removed in a two-step transesterification reaction that is driven by the spliceosome, a large protein-RNA complex. In humans a major and a minor spliceosome exist and are responsible for the removal of two different types of introns. The major spliceosome acts on the majority of introns in humans, U2 type introns. The minor spliceosome acts on a less abundant class of introns, U12 type introns. As the major spliceosome is involved in the splicing of the majority of introns, and is the only type of spliceosome present in *S.cerevisiae*, it is the main focus of research into pre-mRNA splicing, and is discussed below. The major spliceosome is made up of five small nuclear ribonucleoprotein particles, or snRNPs (U1, U2, U4, U5 and U6), each consisting of a small

nuclear RNA (snRNA) in complex with Sm (so called due to their small size) or Sm-like protein (LSm), and snRNP specific proteins (Will and Lührmann, 2001).

1.6 snRNP biogenesis

The U1, U2, U4 and U5 snRNAs are transcribed by RNA polymerase II and gain a 7-methylguanosine (m7G) cap structure before being exported into the cytoplasm where formation of the snRNP is initiated. Three steps in snRNP biogenesis occur in the cytoplasm; Binding of the Sm proteins (B/B', D1, D2, D3, E, F and G) in a ring structure at the conserved Sm binding site of each snRNA, conversion of the m7G cap to a 2,2,7-tri-methylated guanosine (m3G) cap, and trimming of the 3' end of the snRNA to produce the mature length snRNA. A large protein complex present in the cytoplasm, the survival of motor neuron (SMN) complex, is involved in all of these processes. The m3G cap structure and the Sm proteins act as a nuclear localisation signal and snRNPs are imported back into the nucleus. Once back in the nucleus pseudouridylation and 2'-O-methylation occurs at specific sites of each snRNA. The final step in snRNP biogenesis is binding of the snRNP specific proteins. It is thought that sub-organelles within the nucleus, known as Cajal Bodies, which consist largely of protein and RNA, are involved in the pseudouridylation and 2'-O-methylation events, and may be the site of addition of the snRNP specific proteins to the snRNAs (Patel and Bellini, 2008; Will and Lührmann, 2001). Assembled snRNPs are thought to be stored in interchromatin granules within the nucleus until they are required for spliceosome assembly at the active sites of transcription (Malatesta et al., 1999; Patel and Bellini, 2008). Biogenesis of the U6 snRNP is different. The U6 snRNA is transcribed by RNA polymerase III and biogenesis of the U6 snRNP appears to take place in the nucleus. The U6 snRNA is also subjected to pseudouridylation and 2'-O-methylation. The U6 snRNA is not bound by Sm

proteins, instead it binds seven Sm-like proteins (LSm2, 3, 4, 5, 6, 7 and 8), which form a ring structure. This is followed by association of U6 snRNP specific proteins at Cajal Bodies and storage in interchromatin granules (Malatesta et al., 1999; Patel and Bellini, 2008; Will and Lührmann, 2001).

Knowledge of snRNP assembly in yeast in very limited, although it is known that yeast do not contain an apparent SMN complex. The biogenesis of snRNPs in yeast is likely to be the focus of research in the future.

1.7 Spliceosomal assembly and activation in *S.cerevisiae*

For pre-mRNA splicing to occur, the spliceosome has to assemble and undergo several major rearrangements. This process is outlined below and in Figure 1.3.

1.7.1 U1 and the 5' splice site

The first step of spliceosomal assembly is U1 snRNP contacting the 5' exon of the pre-mRNA (Figure 1.3) (Ruby and Abelson, 1988; Seraphin and Rosbash, 1989). Conserved nucleotides at the 5' end of the U1 snRNA base-pair with nucleotides of the intron and exon spanning the 5' splice site (Seraphin et al., 1988; Siliciano and Guthrie, 1988; Zhuang and Weiner, 1986). This base-pairing and anchoring event requires many proteins and is independent of the branch point sequence and ATP (Seraphin and Rosbash, 1991). This is an important step in early spliceosome assembly and marks commitment to spliceosome assembly and pre-mRNA splicing (Seraphin and Rosbash, 1989; Seraphin and Rosbash, 1991). This complex is known as the E complex. Following U1 snRNP binding, the Branch point Bridging Protein (BBP) recognises and binds the branch point in a sequence specific manor. BBP then interacts with

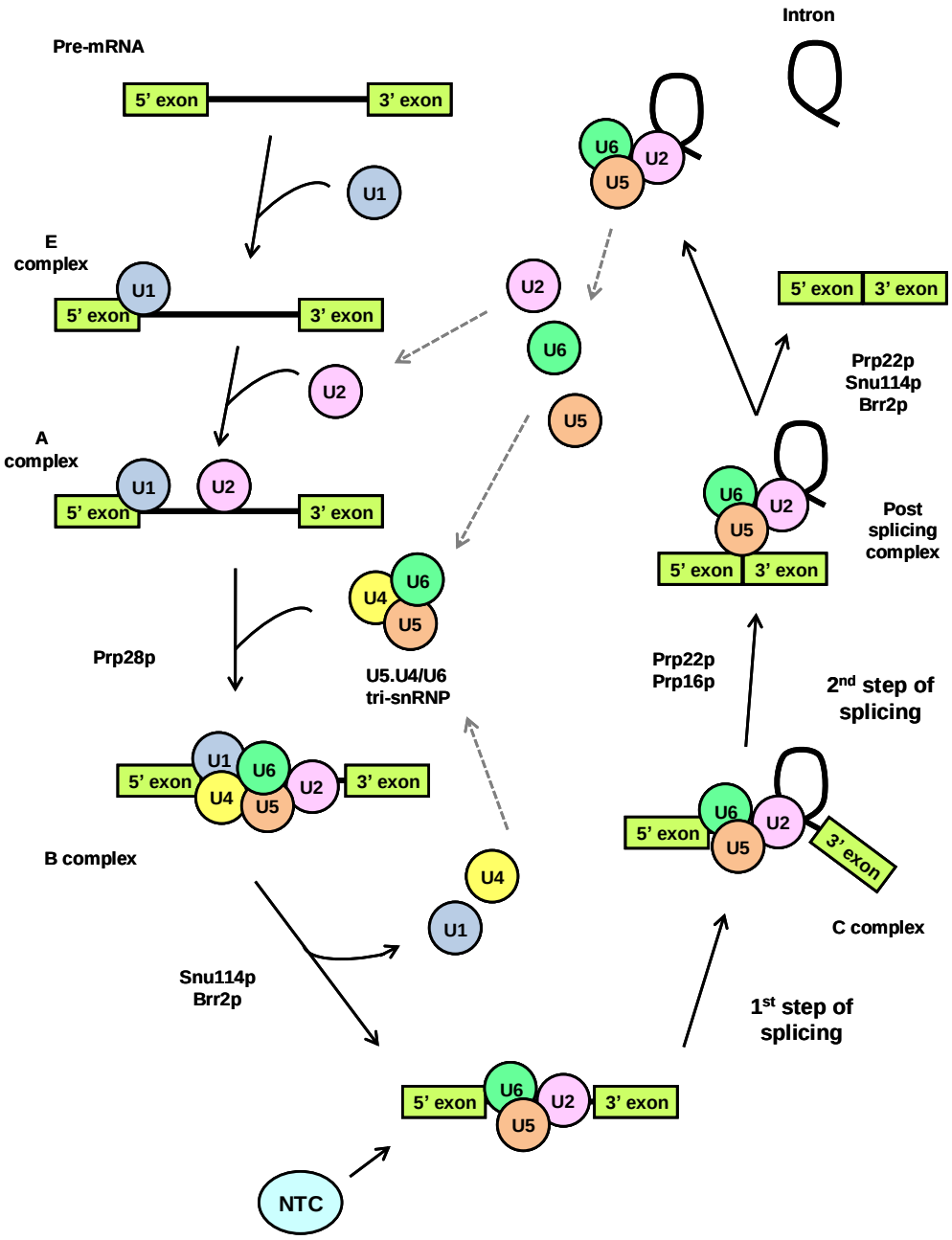


Figure 1.3 Spliceosome assembly and activation. Pre-mRNA splicing is catalysed by the spliceosome. The spliceosome assembles in a step wise manner and undergoes several rearrangements to enable the two catalytic steps of pre-mRNA splicing. The major events in the spliceosome assembly and activation, including snRNPs contacting the pre-mRNA and the Prp19 complex (NTC) joining, are outlined here. Dashed arrows indicate recycling of snRNPs. The timing of the involvement of specific proteins including several ATPases is also illustrated. Figure adapted from Will and Lührmann, 2005.

Mud2p which binds the polypyrimidine tract (Abovich and Rosbash, 1997; Berglund et al., 1997). Mud2p also interacts with the U1 snRNP protein Prp40p bringing the 5' and 3' ends of the intron close together (Zamore and Green, 1989).

1.7.2 U2 and the branch point region

Once BBP has bound to the pre-mRNA, the U2 snRNP also contacts the pre-mRNA, recognising and base-pairing with the branch point region (Figure 1.3) (Berglund et al., 1997; Cellini et al., 1986; Parker et al., 1987). This forms a complex termed the A complex. The branch point region is made up of the conserved sequence UACUAAC, the last adenosine residue, known as the branch nucleotide, is essential for the splicing reaction and is bulged out of the pre-mRNA when U2 snRNA is bound (Query et al., 1994). This step of spliceosome assembly is ATP dependent and requires several proteins to associate with U2, including Prp9, Prp11 and Prp21, along with a stem-loop structure in U2 (stem loop IIA) which is required for the interaction between U2 and the pre-mRNA (Ares and Igel, 1990; Ruby et al., 1993). Recent evidence has supported the theory that there is a branch point-interacting stem-loop (BSL) that presents the nucleotides of the U2 snRNA, is involved in recognition of the branch point, and is disrupted as the interaction between the U2 snRNA and the branch point is formed (Perriman and Ares Jr., 2010).

1.7.3 U5.U4/U6 tri-snRNP binding

Following U1 and U2 snRNPs contacting the pre-mRNA, the next step in spliceosome assembly is ATP dependent recruitment of the U5.U4/U6, or tri-snRNP complex (Figure 1.3). This forms the B complex. In the tri-snRNP the U4 and U6 snRNAs are extensively base paired together, with over 20 base pairs being formed (Hashimoto and Steitz, 1984; Madhani

and Guthrie, 1992). Upon integration of the tri-snRNP into the spliceosome, U5 snRNA interacts with nucleotides in the 5' exon, next to the 5' splice site (Newman et al., 1995; Wyatt et al., 1992).

1.7.4 U4/U6 duplex unwinding and the release of U1 and U4 snRNPs

As the tri-snRNP joins the spliceosome, the U4/U6 interaction becomes destabilised and the two snRNAs unwind. This requires ATP, along with Brr2p, a DExD/H box ATPase, Snu114p which is a GTPase and Prp8p (Fabrizio et al., 1997; Kim and Rossi, 1999; Kuhn et al., 1999). The conserved Loop 1 of the U5 snRNA interacts with the 5' exon and the base pairing between the U1 snRNA and the intron becomes destabilised (Alvi et al., 2001). This destabilisation of the U1 snRNA/5' splice site interaction requires an ATPase, Prp28p (Staley and Guthrie, 1999). It is thought that Prp28p destabilises protein components of the U1 snRNP (Chen et al., 2001; Staley and Guthrie, 1999). At this point, the U6 snRNA base-pairs with the conserved intronic sequence next to the 5' splice site that U1 was previously paired with, and the U1 snRNP dissociates from the spliceosome (Madhani and Guthrie, 1994) (Figure 1.3). Once the unwinding of U4/U6 is complete, the Prp19p-associated complex (NineTeen Complex, or NTC) associates with the spliceosome, stabilising the interactions of U5 and U6 snRNAs with the pre-mRNA (Chan et al., 2003).

A U5 snRNP protein, Prp8p is thought to inhibit the activities of Prp28p and Brr2p until the spliceosome has formed, at which point the inhibition is relieved, allowing the structural rearrangements required for spliceosomal activation to take place (Kuhn et al., 2002). The complex remaining following the release of the U1 and U4 snRNPs, contains the U2, U5 and U6 snRNPs and is termed the C complex (Figure 1.3).

1.7.5 U2-U6 helices

To promote the two steps of splicing the U2 and U6 snRNAs form several helices at the catalytic core of the spliceosome that bring together the reactive regions of the pre-mRNA to allow splicing to occur. These helices are able to form once the U4/U6 duplex has unwound and U4 snRNP is released (Figure 1.4) (Lamond et al., 1988; Sashital et al., 2004). During the formation of the U2-U6 complex a conserved sequence in U6 interacts with sequences in U2 snRNA, forming Helix I (Fabrizio and Abelson, 1990). The region of U2 snRNA involved in formation of Helix I is just upstream of the nucleotides in U2 snRNA that interact with the branch point. The nucleotides of U6 snRNA present in Helix I are adjacent to U6 snRNA nucleotides that interact with the 5' splice site. Therefore, the formation of the U2-U6 helix I and its binding to the pre-mRNA brings together the nucleophile (2'OH) for the first splicing step, the branch point adenosine and the phosphate to be attacked at the 5' splice site (Madhani and Guthrie, 1992). Helix II involves base-pairing between the 5' end of the U2 snRNA and the 3' end of U6 (McPheeters and Abelson, 1992). Residues of the U6 snRNA that were previously base-paired with the U4 snRNA form an intramolecular stem-loop (ISL) (Fortner et al., 1994). The U6 ISL contains a metal binding site which is required for cleavage at the 5' splice site (Sashital et al., 2004; Yean et al., 2000). The U2 snRNA forms a stem-loop structure (stem-loop 1), which with helix I, helix II and the U6 ISL form a four helix junction (Sashital et al., 2004). This four helix junction acts as a scaffold to bring together reactive regions of the pre-mRNA for the first step of splicing (Madhani and Guthrie, 1992). For the second step of splicing Helix II and U6 ISL are still present, but the U2 stem-loop 1 is disrupted and Helix I is split into two halves, Ia and Ib, separated by a to nucleotide bulge in U2 snRNA (Madhani and Guthrie, 1992).

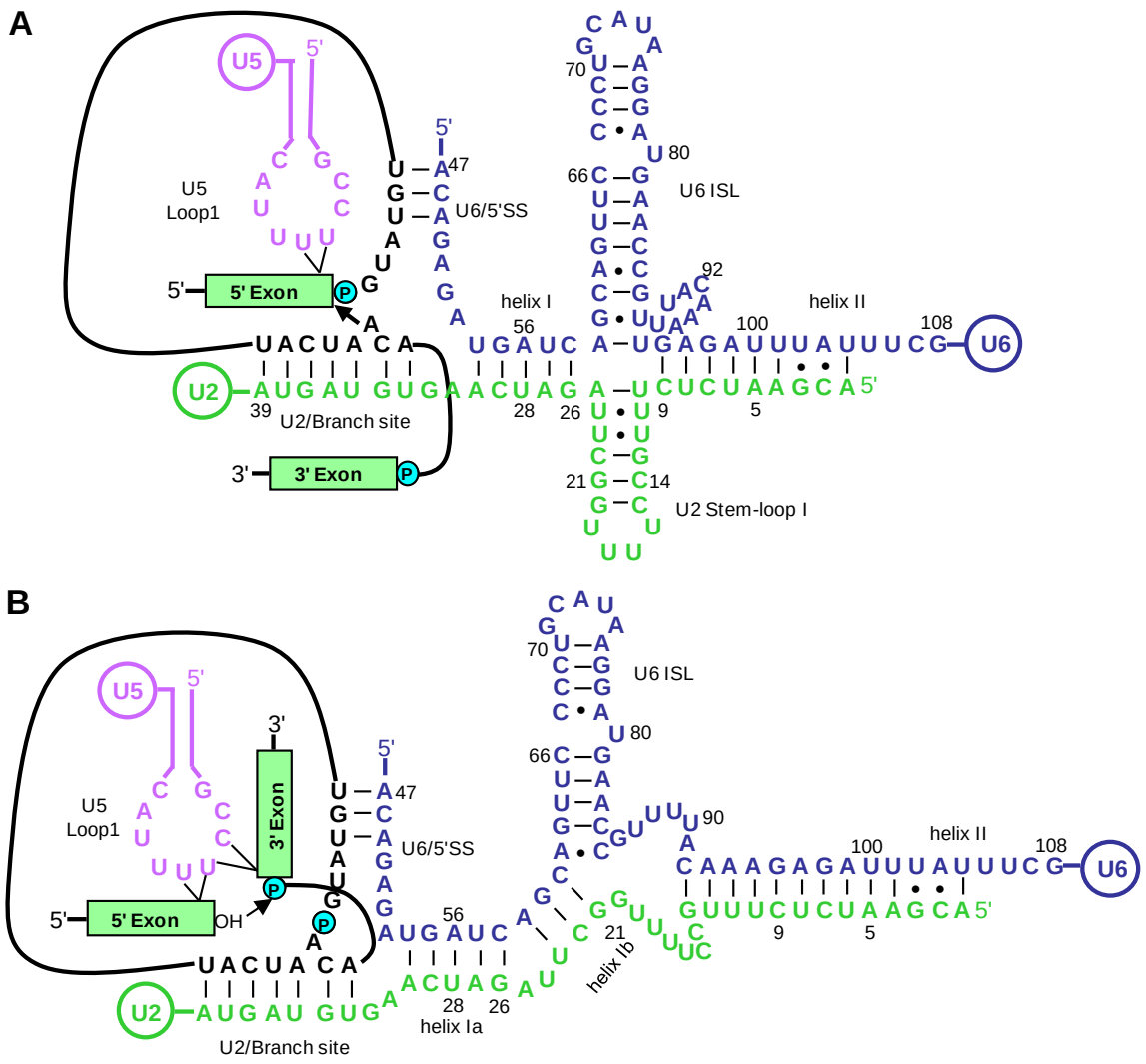


Figure 1.4 U2-U6 helices within the catalytically active spliceosome in *S.cerevisiae*. A. Figure illustrating the helices formed by U2 and U6 snRNAs (helix 1, helix II, U2 stem loop 1 and U6 internal stem loop or ISL) for the first step of splicing to occur. Also illustrated are the interactions of the pre-mRNA and U5 Loop 1, U2 snRNA and the U6 snRNA. The black arrow indicates the branch point adenosine attack of the 5' splice site phosphate. B. Figure illustrates the helices formed by U2 and U6 snRNAs (helix 1a and 1b) for the second step of pre-mRNA splicing. Also illustrated are the interactions of the pre-mRNA and U5 Loop 1, U2 snRNA and the U6 snRNA. The black arrow indicates the 5' exon attack of the 3' splice site phosphate.

1.7.6 U5 snRNA contacts both the 5' and 3' exons to align them for the second step of splicing

U5 interacts with nucleotides in the 5' exon upon integration of the tri-snRNP into the spliceosome (Newman et al., 1995; Wyatt et al., 1992). U5 also contacts nucleotides in the 3' exon, just downstream of the 3' splice site, after the first step of splicing (Newman and Norman, 1992). This allows the 5' exon to be tethered within the spliceosome after the first splicing step and aligns the two exons for the second step of splicing (Newman and Norman, 1992; O'Keefe and Newman, 1998). U1 and U6, which both recognise conserved sequences in introns are thought to position the U5 snRNA, to aid interactions with both the 5' and 3' exons, on the pre-mRNA (Madhani and Guthrie, 1994; Newman and Norman, 1992; Ruby and Abelson, 1988; Seraphin and Rosbash, 1989). More recent data has also suggested a role for the U2 snRNA in positioning the U5 snRNA with the 3' exon for the second step of splicing (McGrail et al., 2006).

In short, activation of the spliceosome requires disruption of the U1/5' splice site interaction, and disruption of the U4/U6 base-pairing, leading to the release of U1 and U4 snRNPs. With the catalytically active spliceosome containing U2, U5 and U6 snRNP, the two transesterification steps can take place (Figure 1.3). After the two splicing steps the intron lariat is released and the remaining spliceosomal components are disassembled and recycled for use in the next round of splicing (Staley and Guthrie, 1998).

Recent evidence has suggested an alternative model of spliceosomal activation (Stevens et al., 2002). This model suggests a preformed complex containing U1.U2.U4/U6.U5 snRNPs, known as a penta-snRNP, binds to the pre-mRNA. Although the penta-snRNP has

been isolated from yeast and human nuclei, it is not clear if this complex represents a functional 'splicing holoenzyme' (Behzadnia et al., 2006; Stevens et al., 2002).

1.7.7 A two state model of the active spliceosome

It has been proposed that the conformations of the spliceosome that support the first and second steps of splicing exist in an equilibrium, with one state being promoted at the detriment of the other state (Query and Konarska, 2004). Prp16p is involved in the transition between the first and second steps of splicing. Mutations that reduce ATP hydrolysis of Prp16p reduce fidelity of the first step of splicing, due to kinetic proof reading, where Prp16p acts as a timer for changing between the first and second step conformations of the spliceosome (Burgess and Guthrie, 1993). Suboptimal substrates would take longer to undergo the first step of pre-mRNA splicing, so would not complete the first step before the conformational change to the second step conformation of the spliceosome (Valadkhan, 2007). Prp22p is involved in the transition between the second step of splicing and disassembly of the post splicing complex (Mayas et al., 2006). Prp22p controls the transition between the second step conformation and disassembly of the post splicing complex in a similar manner to Prp16p (Valadkhan, 2007). *prp22* alleles have been identified that suppress the transition to disassembly of the post splicing complex and *prp16* alleles have been identified that inhibit the transition to the second step of pre-mRNA splicing. Prp8p mutants that suppress the second step of splicing, exacerbated growth defects of these *prp22* alleles and suppress defects of *prp16* alleles. Prp8p mutants that suppress the first step of splicing, exacerbated growth defects of the *prp16* alleles and suppresses that of *prp22* alleles (Liu et al., 2007). This has led to the proposition that the U5 snRNP protein, Prp8p, controls the shift between the two conformations of the spliceosome (Liu et al., 2007).

1.7.8 snRNP recycling

Following pre-mRNA splicing the excised intron is released; a process that requires Prp22p. Following release of the excised intron, the post splicing complex is disassembled. The components of the disassembled post splicing complex and the released U1 and U4 snRNPs are recycled for use in new rounds of splicing. Prp24p is known to be involved in recycling of snRNPs, and reanneals U4 and U6 snRNAs for use in new rounds of splicing (Raghunathan and Guthrie, 1998).

1.8 The U5 snRNP

1.8.1 Structure of the U5 snRNA

The U5 snRNP is a very important component of the spliceosome as it plays a major role in spliceosome activation and is required for both steps of splicing (Patterson and Guthrie, 1987). The U5 snRNA exists in several complexes; as part of the U5 snRNP, as part of the tri-snRNP, as part of the pre-catalytic spliceosome or penta-snRNP containing all five snRNPs, and as part of the catalytically active spliceosome following the dissociation of U1 and U4 snRNPs (Newman, 1997; Stevens et al., 2002). The U5 snRNA is the only snRNA known to be present in both the U2 (major) and U12 (minor) spliceosomes (Tarn and Steitz, 1996). U5 snRNA in yeast is 214 nucleotides in length and has an evolutionarily conserved secondary structure consisting of two stem loop structures, a 5' and a 3' stem loop, which flank an Sm core protein binding site (Figure 1.5). The 5' stem loop is divided into seven domains; Loop 1, Stem 1 (S1), Internal Loop 1 (IL1), Stem 2 (S2), Variable Stem-Loop (VSL), Internal Loop 2 (IL2) and Stem 3 (S3) (Figure 1.5). Loop 1 contains a nine nucleotide highly conserved sequence (GCCUUUUAC), the 3' side of IL1 is also highly conserved, whereas the 5' side of IL is only

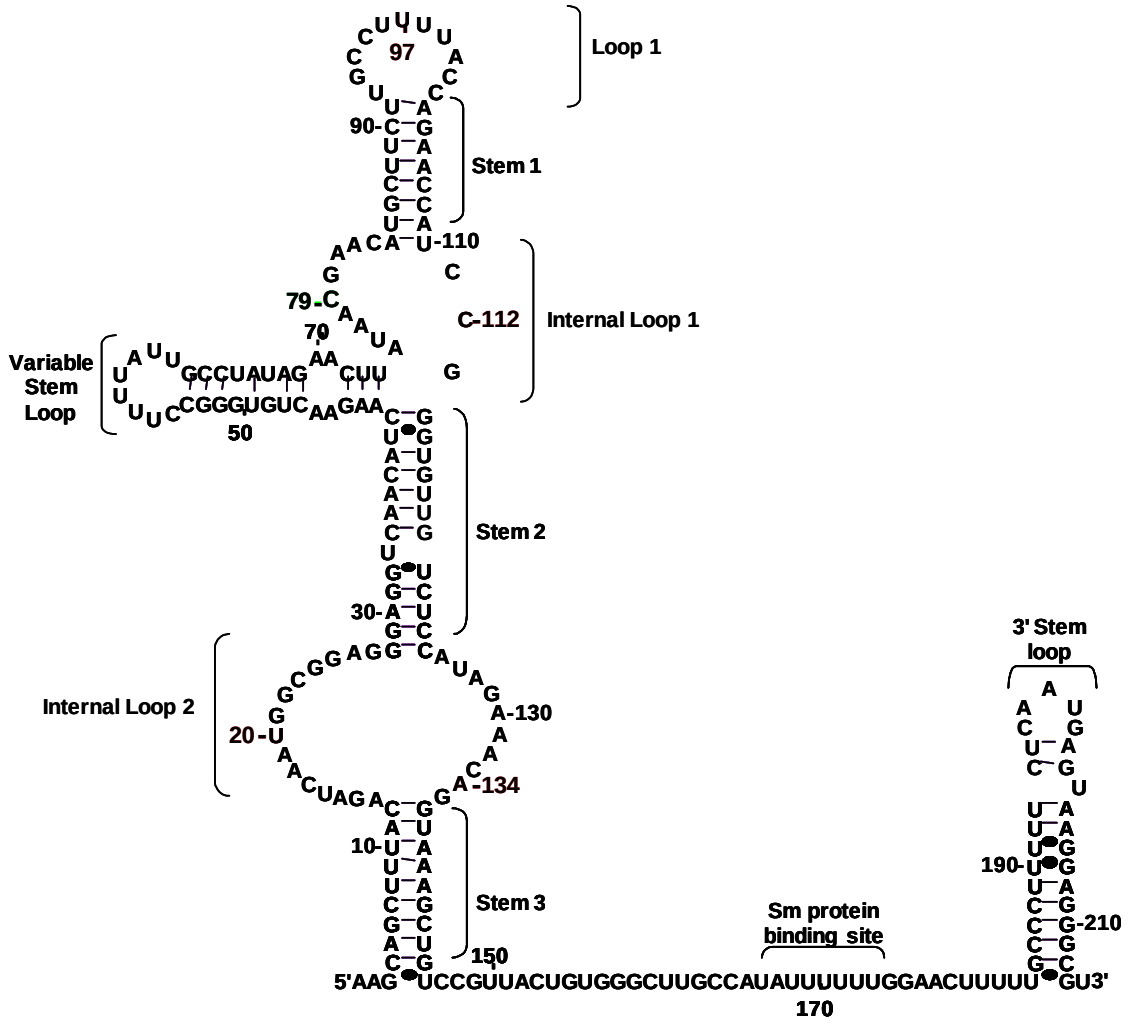


Figure 1.5 Secondary structure of the *S.cerevisiae* U5 snRNA. The U5 snRNA contains a secondary structure consisting of two stem loop structures, a 5' and a 3' stem loop, which flank an Sm core protein binding site. The 5' stem loop is further divided into seven domains:- Loop 1, Stem 1, Internal Loop 1, Varaible Stem Loop, Stem 2, Internal Loop 2 and Stem 3. Figure adapted from Frank *et al*., 1994.

moderately conserved with only nucleotides C79 and G80 being invariant (Frank et al., 1994; Patterson and Guthrie, 1987). Studies of the human U5 snRNA have shown Loop 1 to be important for splice site selection, binding of the U5 snRNP protein U5-220kD or hPrp8p and assembly of the tri-snRNP (Cortes et al., 1993; Hinz et al., 1996; Urlaub et al., 2000). The VSL is absent in mammalian U5 snRNA and is not essential for U5 snRNA function in yeast (Frank et al., 1994). The size and sequence of IL2 is highly divergent (Frank et al., 1994). The Sm site, required for binding of the Sm proteins, is present in all known U5 species, reflecting the importance of this region (Frank et al., 1994). Loop 1, S1, IL1, S2 and the Sm site are all required for U5 function in *S.cerevisiae* (Frank et al., 1994). A shorter, functional form of the U5 snRNA (U5S) also exists in yeast, lacking the 3' stem loop, indicating a greater importance for the 5' stem loop (Patterson and Guthrie, 1987). The importance of the 5' stem loop has also been demonstrated in human cells, with the 5' stem loop containing all the U5 snRNA specific sequence elements that are essential for splicing (Hinz et al., 1996). In humans both of the internal loops, the Sm binding site and the 3' stem loop are required for efficient expression of the U5 snRNA (Hinz et al., 1996).

1.8.2 Protein binding of the U5 snRNA

The U5 snRNP in *S.cerevisiae* consists of the U5 snRNA in a complex with seven Sm proteins (B/B', D1, D2, D3, E, F and G) which are common to U1, U2, U4 and U5 snRNPs, and a set of U5 snRNP specific proteins, including Prp8p, Snu114p, Brr2p, Prp28p, Snu16p and Snu40p (Dib1p) (Lührmann et al., 1990; Mougin et al., 2002). In humans the U5 snRNP contains U5 specific proteins of 15, 40, 52, 100, 102, 110, 116, 200 and 220 kDa (Will et al., 1993). The 15, 52, 100, 116, 200 and 220kD proteins all have *S.cerevisiae* homologues, namely Snu16p, Snu40p, Prp28p, Snu114p, Brr2p and Prp8p, respectively (Anderson et al., 1989; Fabrizio et

al., 1997; Lauber et al., 1996; Mougin et al., 2002). Prp8p, along with Snu114p and the Sm proteins, form a common scaffold for U5 snRNP between humans and yeast (Gottschalk et al., 2001b). Prp8p is the major U5 snRNP specific protein in *S.cerevisiae* and associates with both the U5 snRNA and the tri-snRNP (Garcia-Blanco et al., 1990; Lossky et al., 1987). Prp8p makes contact with both the 5' and 3' splice sites and contacts at least five different regions of the 5' stem-loop of U5 snRNA and is proposed to form the active site at the core of the spliceosome (Abelson, 2008; Dix et al., 1998). The interaction between Prp8p and the U5 snRNA requires both internal loops of the 5' domain of U5 snRNA (Dix et al., 1998). Curiously, Prp8p crosslinks most strongly to a region of U5, the conserved Loop 1, that is not essential for the association of Prp8p with U5 (Dix et al., 1998; Urlaub et al., 2000). In humans the 5' stem loop also appears to be important for association of hPrp8p (Hinz et al., 1996). Snu114p, which at least contacts U5 snRNA at the 5' side of internal loop 1 at position 79, is the only GTPase known to be involved in pre-mRNA splicing (Dix et al., 1998; Fabrizio et al., 1997). Brr2p is a member of the DEXD/H box ATPase family (Lauber et al., 1996; Lin and Rossi, 1996; Noble and Guthrie, 1996; Schwer, 2001; Xu et al., 1996). The DEXD/H box ATPase family of proteins are often known as RNA helicases, and can also act as RNPases disrupting RNA-protein complexes present in the spliceosome (Bleichert and Baserga, 2007; Schwer, 2001). Brr2p and Snu114p are both involved in the unwinding of the U4/U6 snRNA duplex, and the subsequent release of U4 from the spliceosome (Bartels et al., 2002; Staley and Guthrie, 1998). Prp8p is thought to play a role in the regulation of Snu114p and Brr2p activity (Bartels et al., 2003; Kuhn et al., 1999; Kuhn et al., 2002). Prp28p is a DEAD box ATPase protein, and phosphorylation of human Prp28p has been shown to be required for the U4/U6.U5 tri-snRNP particle to assemble into the spliceosome (Mathew et al., 2008; Staley and Guthrie, 1999). Snu16p is an essential U5 snRNP protein whereas Snu40p is non-essential

but has been linked to chromosome segregation, pre-mRNA splicing and DNA replication (Bialkowska and Kurlandzka, 2002). Prp28p and Snu40p are not present in the tri-snRNP. Although the protein composition of the U5 snRNP is well documented little is known about how the U5 snRNP is assembled, especially in yeast. It is known that Prp8p and Snu114p interact directly with the U5 snRNA (Dix et al., 1998), but it is not known if the interactions between other U5 snRNP proteins and the U5 snRNA are direct or indirect, or how the proteins are recruited to the snRNA.

1.8.3 The U5 snRNP in the tri-snRNP

The U5 snRNP is present in the U4/U6.U5 tri-snRNP particle, along with the U4 and U6 snRNPs (Black and Pinto, 1989). The U4 and U6 snRNAs are extensively base-paired together within the tri-snRNP particle (Bringmann et al., 1984; Rinke et al., 1985). Chemical probing, including treatment with DMS and RNase enymes, of the U5 snRNA within the U5 snRNP and the tri-snRNP revealed very little differences in the accessibility of nucleotides of the U5 snRNA, indicating that recognition of U5 snRNA sequences is not requited for the ATP dependent interaction of U5 snRNP with the U4/U6 snRNPs (Black and Pinto, 1989; Mougin et al., 2002). This also suggests that the U5 snRNA stays associated with the U5 snRNP proteins that are present in the tri-snRNP, when it is in complex with U4 and U6 snRNPs (Black and Pinto, 1989; Mougin et al., 2002). In the human U5 snRNP, Loop 1 and IL1 (IL2 in yeast) of the U5 snRNP are involved in tri-snRNP formation (Hinz et al., 1996). Electron cryomicroscopy of human tri-snRNPs has revealed that Loop 1 and the U5 snRNA are located at the centre of a tetrahedrally shaped tri-snRNP particle (Sander et al., 2006). The U5 snRNP proteins Prp8p, Snu114p, Brr2p and Snu16p are all present in the tri-snRNP, along with the Sm proteins (Mougin et al., 2002). The U6 snRNP specific Lsm proteins (Lsm2, 3, 4,

5, 6, 7 and 8) are also present in the tri-snRNP (Gottschalk et al., 1999; Mougin et al., 2002), along with Prp6p, Snu66p, Prp31p, Prp3p, Prp4p, Prp38p, Snu23p, Snu13p, Spp381p and Prp18p (Gottschalk et al., 1999; Mougin et al., 2002; Stevens and Abelson, 1999). Prp18p is a non-essential tri-snRNP protein that is loosely associated with the U5 snRNP, required for only the second splicing step (Horowitz and Abelson, 1993). Recent evidence suggests that a conserved loop present in Prp18p stabilises the interaction between U5 snRNA Loop 1 and the pre-mRNA after the first step of splicing (Bacikova and Horowitz, 2005; Crotti et al., 2007).

1.8.4 Function of the U5 snRNA within the spliceosome

U5 snRNP is required for both steps of pre-mRNA splicing (Patterson and Guthrie, 1987). It is known that Loop 1 of U5, which contains a conserved sequence (GCCUUUUAC) is not essential for the first step of splicing (O'Keefe et al., 1996). The size of Loop 1 is critical for the alignment of the two exons for the second step of splicing (O'Keefe and Newman, 1998). It has also been shown that deletion of Loop 1 abolishes crosslinking between the U5 snRNA and the exons (O'Keefe et al., 1996). Loop 1 of the U5 snRNA contacts non-conserved nucleotide sequences in both exons, first at the 5' splice site, then following the first step of splicing, at the 3' splice site, holding the substrate pre-mRNA in position at the catalytic core of the spliceosome for the second transesterification step to occur (Figure 1.6) (Frank et al., 1994; Newman and Norman, 1992; Siatecka et al., 1999; Sontheimer and Steitz, 1993; Teigelkamp et al., 1995). This is supported by evidence demonstrating the ability of a region of U5 snRNA Loop 1 to form base-pairing interactions with other nucleic acid (Black and Pinto, 1989). U96, U97 and U98 of U5 Loop 1 have been shown to interact with positions -1, -2 and -3 in the 5' exon, just before the 5' splice site, and U5 Loop 1 nucleotides C95 and U96 interact with positions +1 and +2 of the 3' exon (Cortes et al., 1993; Newman and Norman,

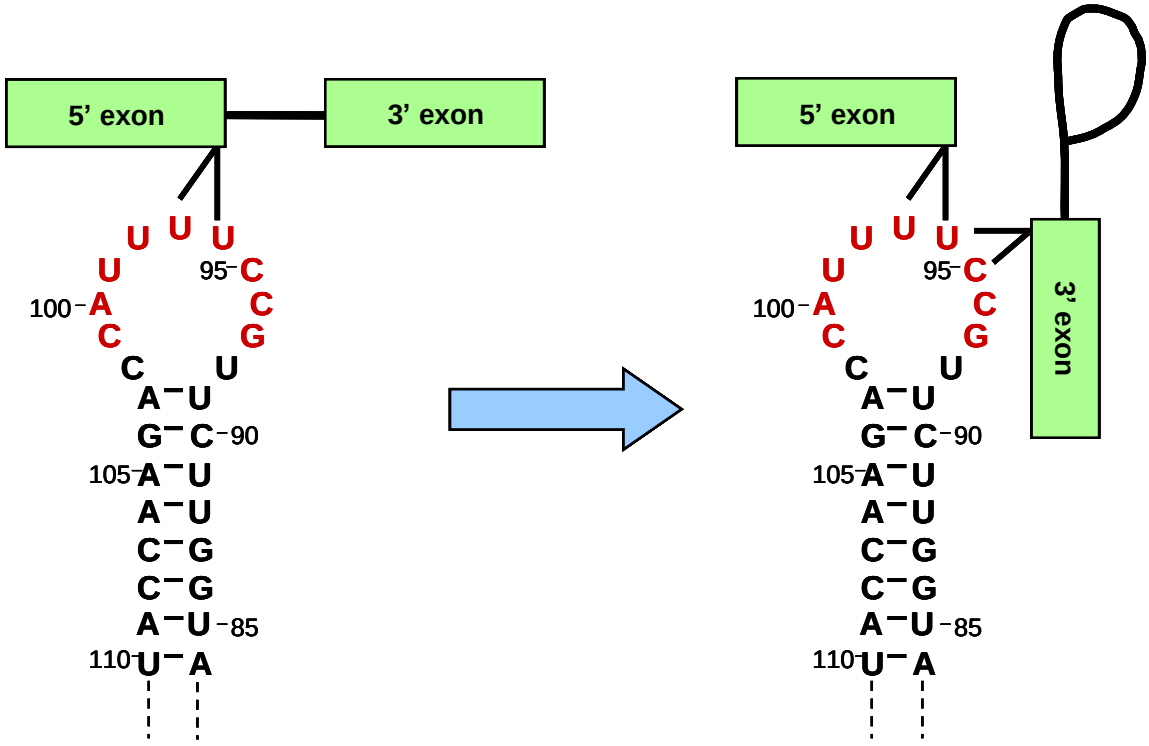


Figure 1.6 The role of U5 snRNA Loop 1 in exon alignment, facilitating the two steps of pre-mRNA splicing. U5 snRNA Loop 1 anchors the 5’ exon at the catalytic core of the spliceosome for the first step in pre-mRNA splicing, and anchors both the 5’ and 3’ exons for the second step. The conserved nucleotides of U5 Loop1 are highlighted in red. Dashed lines indicate that RNA structure shown here is only part of the U5 snRNA.

1992; Newman et al., 1995; Sontheimer and Steitz, 1993). Evidence suggests that these exon sequences are first contacted by the U5 snRNP protein Prp8p, which is thought to be the principle factor in anchoring exons to the spliceosome (Beggs et al., 1995; Dix et al., 1998; Siatecka et al., 1999; Teigelkamp et al., 1995). Prp8p appears to hold U5 Loop 1 and the exons at the catalytic centre of the spliceosome for the catalytic splicing steps to occur (Beggs et al., 1995). The U5 snRNA also positions several essential U5 snRNP proteins that are required for pre-mRNA splicing, including the GTPase Snu114p, to the catalytic core of the spliceosome (Dix et al., 1998). U5 snRNA also exists in a complex with the splicing factor Aar2p, the Sm proteins, Snu114p and Prp8p (Gottschalk et al., 2001a). The structure of this complex is similar to that of the human U5 snRNP, indicating that Sm proteins, Snu114p and Prp8p form the basic structure of the U5 snRNP (Gottschalk et al., 2001a). Aar2p is not present in the U4/U6.U5 tri-snRNP, but is thought to be involved in snRNP recycling (Gottschalk et al., 2001a).

1.9 The three major U5 snRNP proteins- Prp8p, Snu114p and Brr2p

1.9.1 The U5 snRNP protein, Prp8p

Prp8p, at 280 kDa, is the largest protein component of the yeast spliceosome. Prp8p and its role in pre-mRNA splicing, were identified in a screen for temperature sensitive mutants with defects in pre-mRNA splicing (Jackson et al., 1988; Lin et al., 1987; Lustig et al., 1986; Vijayraghavan et al., 1986). Prp8p associates with both the U5 snRNP and the U4/U6.U5 tri-snRNP, is present in the active spliceosome and persists through to the post splicing complex (Garcia-Blanco et al., 1990; Lossky et al., 1987; Whittaker et al., 1990). Prp8p has been shown to crosslink to Loop 1 and both internal loops of the U5 snRNA, which are essential for Prp8p association with the U5 snRNA (Dix et al., 1998). Prp8p is a homologue of the human U5

snRNP protein, U5-220kD or hPrp8p, which is known to be present in both the U2 and U12 dependent spliceosomes (Luo et al., 1999).

1.9.1.1 Structure and domains of Prp8p

Although Prp8p is highly conserved across different organisms it bears no striking homology to any other proteins (Grainger and Beggs, 2005). However, several recognisable motifs and domains have been identified within Prp8p. These include a nuclear localisation signal (NLS), RNA recognition motif (RRM), MPN/Jab1 domain, RNase H domain and a β-finger domain (Figure 1.7) (Grainger and Beggs, 2005; Pena et al., 2009; Pena et al., 2008; Yang et al., 2008). The NLS is present at the N-terminus of Prp8p, is sufficient for Prp8p localization in the nucleus and is thought to play a role in U5 snRNP maturation (Boon et al., 2007). Several mutations within the RRM of Ppr8p suppress the cold sensitive phenotype of specific U4 snRNA and *brr2* mutants, which effect U4/U6 duplex unwinding (Kuhn and Brow, 2000; Kuhn et al., 1999; Kuhn et al., 2002). This suggests a role for the RRM of Prp8p in U4/U6 unwinding (Grainger and Beggs, 2005). The MPN/Jab1 domain (amino acids 2178-2310) is present in the C-terminus of Prp8p, and has been shown to be important for the ability of Prp8p to bind ubiquitin (Bellare et al., 2006; Grainger and Beggs, 2005; Pena et al., 2007). Mutations in the MPN/Jab1 domain of Prp8p that effect ubiquitin binding also reduce splicing efficiency and tri-snRNP formation, supporting a role for ubiquitination in splicing (Bellare et al., 2006; Bellare et al., 2008). Prp8p contains a truncated RNase H domain which lies near a 5' splice site interacting region of Prp8p (Pena et al., 2008). This RNase H domain is thought to be involved in stabilizing the core of the spliceosome and takes part in the rearrangements occurring there, that are required for the two catalytic steps of splicing (Pena et al., 2008). This domain contains the β-finger domain (amino acids 1822-2095) present in the C-terminus of

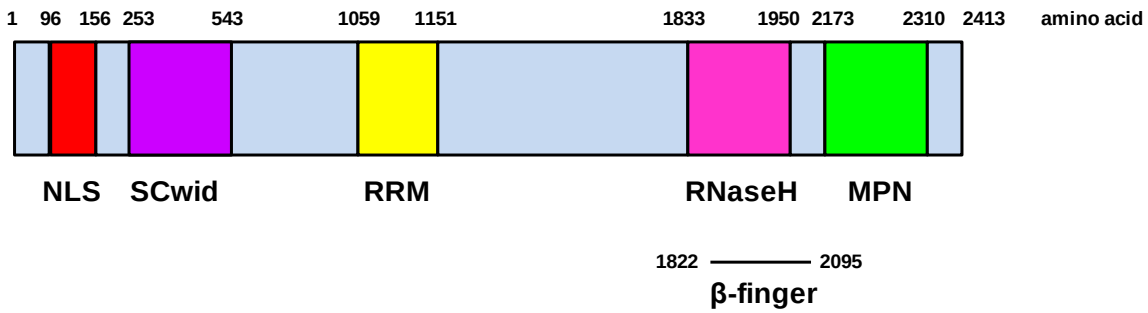


Figure 1.7 The domains of *S.cerevisiae* Prp8p. Several domains have been identified within Prp8p including a nuclear localisation signal (NLS), an RNA recognition motif (RRM), an RNAase H domain and the overlapping β-finger structure. Prp8p also contains an MPN/Jab1 domain and a Snu114p and Cwc21p interacting domain (SCwid).

Prp8p (Yang et al., 2008). Mutations in the β-finger affect the equilibrium between the first and second steps of splicing, and U4/U6 duplex unwinding (Yang et al., 2008). A protein interaction domain of Prp8p has recently been identified as a site of Snu114p and Cwc21p interaction (Grainger et al., 2009). This domain has been termed the SCwid domain (Snu114p and Cwc21p Interacting Domain) and is present in the N-terminus of Prp8p (Grainger et al., 2009). The discovery of the interaction between Prp8p and the NTC protein Cwc21p, has led to the proposition that Cwc21p may have a regulatory role at the catalytic core of the spliceosome (Grainger et al., 2009). Yeast two-hybrid data has also suggested the presence of an intramolecular fold between amino acids 342 and 544 of Prp8p (Grainger et al., 2009; Kuhn and Brow, 2000).

1.9.1.2 Prp8p is situated at the catalytic core of the spliceosome

Prp8p is known to be a key U5 snRNP and U4/U6.U5 tri-snRNP protein and interacts directly with the U5 snRNA, with crosslinks between Prp8p and the U5 snRNA being detected on both side of Internal Loops 1 and 2, and Loop 1 of the U5 snRNA (Dix et al., 1998; Urlaub et al., 2000). Two regions of Prp8p (amino acids 770-871 and 1281-1431) have been shown to crosslink to position 97 in U5 snRNA Loop 1 (Turner et al., 2006). Depletion of U5 snRNA and certain mutations in Loop 1 of U5 snRNA have been shown to reduce stability of Prp8p (Kershaw et al., 2009). Prp8p also interacts directly with the U6 snRNA (Vidal et al., 1999). A crosslink has been identified between Prp8p and position 54 of the U6 snRNA, which is in a conserved region of the U6 snRNA, directly downstream of the region of U6 that contacts the 5' splice site, known to be involved in formation of the catalytic core of the spliceosome (Vidal et al., 1999). Prp8p also interacts with the pre-mRNA, with crosslinks to the 5' splice site being identified before and after the first step of splicing (Reyes et al., 1999; Reyes et al.,

1996; Siatecka et al., 1999; Teigelkamp et al., 1995). Prp8p has also been shown to crosslink to the 3' splice site over a region spreading from the branch site into the 3' exon (Teigelkamp et al., 1995; Umen and Guthrie, 1995). It is thought that Prp8p first crosslinks to the 5' splice site, then also at the 3' splice site for the second step of splicing (Siatecka et al., 1999). Three regions of Prp8p have been shown to be involved in the crosslinks to the 5' splice site and branch site (Turner et al., 2006). These three regions of Prp8p are between amino acids 871-970, 1281-1413 and 1503-1673 (Turner et al., 2006). The second of these regions of Prp8p has also been shown to crosslink to U5 snRNA and the third has been shown to crosslink to the U6 snRNA (Turner et al., 2006). In hPrp8p a five amino acid region (1894-1898) has been identified as the region contacting the conserved GU motif at the 5' splice site (Reyes et al., 1999; Reyes et al., 1996). This evidence alone demonstrates a central position for Prp8p at the catalytic core of the spliceosome.

As well as interacting directly with the U5 and U6 snRNAs, Prp8p interacts genetically with the U2 and U4 snRNAs (Kuhn and Brow, 2000; Xu et al., 1996). Mutations in Prp8p shown to suppress the cold sensitive phenotype of the U4 cs-1 mutant, which exhibits a U4/U6 unwinding defect, map to five regions of Prp8p (Kuhn and Brow, 2000). One of these regions over laps with a region that interacts with Prp40p, a U1 snRNP protein (Abovich and Rosbash, 1997). The fact that Prp8p has some form of interaction with the U1, U2, U4, U5 and U6 snRNA, and both exons indicates that Prp8p is a major component of the spliceosome and plays a vital role in pre-mRNA splicing. Prp8p has also been shown to interact with many pre-mRNA splicing factors, including the U1 snRNP proteins Prp39p, Prp40p and Snp1 (Abovich and Rosbash, 1997; Awasthi et al., 2001; van Nues and Beggs, 2001). Prp8p has also been shown to interact extensively with the U5 snRNP proteins Brr2p and Snu114p (Achsel et al., 1998; Boon et al., 2006; van Nues and Beggs, 2001; Zhang et al., 2009). These interactions

will be discussed in detail later. The vast web of interactions of Prp8p with other protein and snRNAs emphasises both the size of Prp8p and how extensively involved Prp8p is in pre-mRNA splicing.

1.9.1.3 The functions of Prp8p in pre-mRNA splicing

With Prp8p being situated at the core of the spliceosome and being so extensively involved in protein and RNA interactions at the catalytic core, Prp8p is often seen as the platform on which the core is based. But Prp8p is also involved in the rearrangements that occur to allow splicing to occur. Genetic interactions between Prp8p mutants with Prp28p, Brr2p and U6 snRNA mutants, strongly supports a role for Prp8p in activation of the spliceosome (Kuhn et al., 2002). Further to this, a *prp8* mutant was discovered that overcomes a block in U4/U6 duplex unwinding seen in a cold sensitive U4 snRNA mutant (U4-cs1) (Kuhn et al., 1999). This indicates that Prp8p functions during U4/U6 duplex unwinding, along with Snu114p and Brr2p, contributing to activation of the spliceosome (Kuhn et al., 1999). It has since been demonstrated that the C-terminus of Prp8p is required for the ATP dependent unwinding of the U4/U6 duplex by Brr2p (Maeder et al., 2009). Evidence suggests that Prp8p also functions to hold Loop 1 of the U5 snRNA and the two exons, supporting the interactions between the U5 snRNA and the exons, at the centre of the active spliceosome to allow the catalytic steps to occur (Aronova et al., 2007; Beggs et al., 1995). This theory is supported by Prp8p contacting both exons and the extensive interactions between Prp8p and the 5' stem loop of the U5 snRNA, including Loop 1 (Dix et al., 1998; Teigelkamp et al., 1995; Umen and Guthrie, 1995). A specific Prp8p mutant (*prp8*-R1753K) suppresses several Prp22p mutations that cause defects in the helicase activity of Prp22p, which is required for the second step of splicing, and release of mRNA following splicing (Schneider et al., 2004; Schneider et al.,

2002). It is also known that the temperature sensitive phenotype of *prp8*-R1753K can be suppressed by certain mutations in U5 Loop 1 or Prp18p, which is required for positioning the 3' splice site for the second step of splicing (Aronova et al., 2007; Crotti et al., 2007). The Prp18p mutant also suppresses several mutations in Slu7, which has a role in the second step of splicing (Aronova et al., 2007). These data illustrate a link between Prp8p and the second step of splicing, and further supports a role for Prp8p in U5 snRNA/exon interactions (Aronova et al., 2007). A genetic screen for suppressors of a branch site mutation that block splicing between the first and second step of splicing, identified *prp8* mutants as suppressors (Query and Konarska, 2004). These *prp8* mutants suppress mutations at the 3' splice site, 5' splice site and branch site, and improve the second step of splicing, while inhibiting the first step (Query and Konarska, 2004). A second set of *prp8* mutants have since been identified, that suppress defects in the first step of splicing, and improve the first step of splicing while inhibiting the second step (Liu et al., 2007). This led to the proposition that Prp8p controls the equilibrium between the first and second steps of splicing (see 1.9.8 A two state model of the active spliceosome) (Liu et al., 2007).

1.9.1.4 Prp8p and Retinitis Pigmentosa

Several mutations in the C-terminus of hPrp8p have been linked to Retinitis Pigmentosa in humans. The yeast equivalents of several of these mutations are located in the region of the C-terminus of Prp8p, which forms the MPN/Jab1 domain (Boon et al., 2007; Pena et al., 2007). The yeast equivalents of the human Retinitis Pigmentosa linked mutations lead to a defect in maturation of the U5 snRNP and disrupt the interaction between the C-terminus of Prp8p with Snu114p and Brr2p (Boon et al., 2007; Pena et al., 2007). This suggests U5 snRNP maturation as a molecular basis for Retinitis Pigmentosa (Boon et al., 2007).

1.9.2 The U5 snRNP protein, Snu114p

Snu114p is a 114 kDa, *S.cerevisiae* protein which is essential for cell viability and splicing *in vivo* (Fabrizio et al., 1997; Frazer et al., 2008). Snu114p is a GTPase, the only one currently known to be involved in pre-mRNA splicing (Fabrizio et al., 1997). Genetic depletion of Snu114p inhibits pre-mRNA splicing, resulting in accumulation of unspliced pre-mRNA (Fabrizio et al., 1997). Snu114p is a component of the U5 snRNP, contacting U5 RNA at the 5' side of Internal Loop 1 at position C79 (Dix et al., 1998). As well as interacting directly with U5 snRNA, Snu114p has also been shown to interact genetically with the U2, U4 and U6 snRNA (Frazer et al., 2009). This emphasises the importance of Snu114p in pre-mRNA splicing and its extensive interactions within the spliceosome. Snu114p is a homologue of the human U5 snRNP protein U5-116kD, or hSnu114p, which is also evolutionarily conserved across other organisms (Fabrizio et al., 1997). Like Snu114p, hSnu114p also has GTP binding properties, binding GTP when it is part of the U5 snRNP (Fabrizio et al., 1997). hSnu114p is predicted to cause structural rearrangements in the spliceosome, and has been implicated in 3' splice site selection (Fabrizio et al., 1997; Liu et al., 1997). The hSnu114p and its *S.cerevisiae* homologue, Snu114p, are closely related to a ribosomal translocase, known as elongation factor-2 (EF-2) in eukaryotes and EF-G in prokaryotes. Yeast EF-2 is 26% identical and 46% similar to Snu114p (Brenner and Guthrie, 2005). EF-2 (which is encoded by the EFT1 and EFT2 genes in yeast) and Snu114p both contain G domains, made up of elements G1 to G5, containing the consensus sequence elements required for GTP binding and hydrolysis (Fabrizio et al., 1997). EF-2 is known to be involved in structural rearrangements during translocation in the ribosome, so it is thought that Snu114p will perform a role similar to this in the spliceosome (Dix et al., 1998; Fabrizio et al., 1997; Staley and Guthrie, 1998).

1.9.2.1 Structure and domains of Snu114p

As Snu114p share sequence similaities with the ribosomal elongation factor EF-2 it has been divided into protein domains based on those of EF-2 (Figure 1.8) (Brenner and Guthrie, 2005; Fabrizio et al., 1997). EF-2 contains the domains I, II, III, IVa, V and IVb, with domain I containing the conserved G domain motifs, and domains IVa and IVb comprising the C-terminal domain. The structure of EF-2 has been solved by X-ray crystallography revealing that domains I and II exist as a rigid unit, with domain III rotating around the region between domains II and III. Domains IV and V also rotate as a rigid unit (Figure 1.8) (Jorgensen et al., 2002; Jorgensen et al., 2003). This structure is flexible and changes in conformation upon GTP binding (Jorgensen et al., 2003). Snu114p contains the domains I, II, III, IVa, V and IVb, like EF-2, but also contains an N terminal domain that is not present in EF-2 (Figure 1.8) (Fabrizio et al., 1997). Domain I contains GTPase motifs (G1 to G5) and a region known as G'', which is unique in both Snu114p and EF-2. Domains IVa and IVb are thought of as a single domain, the C-terminal domain, with domain IVb being predicted to fold back and interact with domain IVa (Brenner and Guthrie, 2005). The unique N-terminus of Snu114p is aspartic and glutamic acid rich and appears to be important for activation of the spliceosome (Bartels et al., 2002). Deletion of the N-terminal domain results in a temperature sensitive phenotype, with efficient U4 snRNA release during spliceosomal activation being prevented at the restrictive temperature, causing spliceosomal arrest (Bartels et al., 2002). At the restrictive temperature U4 snRNA was still base paired with U6 RNA, demonstrating the role of the N-terminal region of Snu114p in the unwinding of U4/U6 snRNAs during spliceosomal activation (Bartels et al., 2002; Staley and Guthrie, 1998). The N-terminal deletion does not prevent Snu114p binding to the tri-snRNP, suggesting this N-terminal region is required for activation rather than assembly of the spliceosome (Bartels et al., 2002). Deletion of the N-

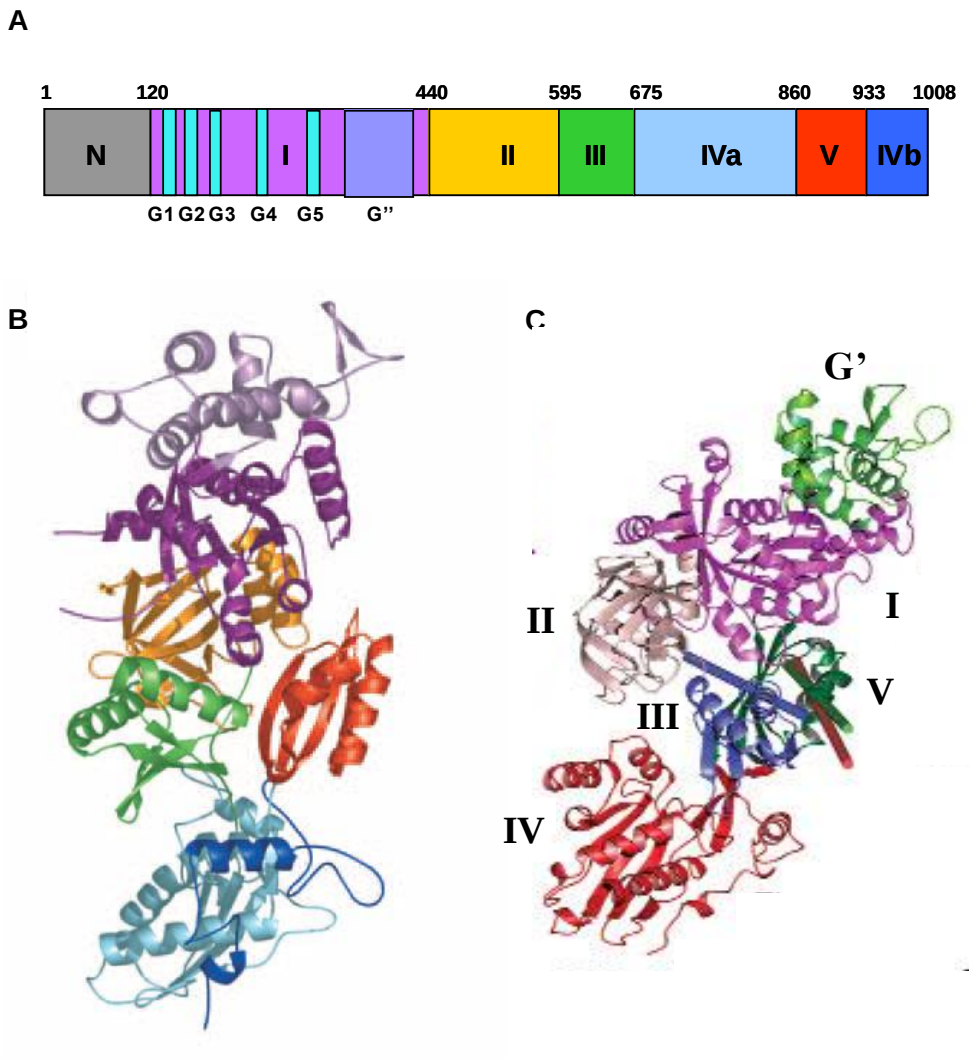


Figure 1.8 The domains of *S.cerevisiae* Snu114p. A. Snu114p is divided into domains I, II, III, IVa, V and IVb based upon sequence similarities with EF-2. Domain I, the G domain, contains the conserved elements G1 to G5 for GTP binding and hydrolysis, and domain G'' which is unique in EF2 and Snu114p. Snu114p also contains a unique N-terminal domain (1-120) that is absent in EF-2. B. The ribbon diagram illustrated the predicted structure of Snu114p based upon sequence similarities with EF2 (predicted structure from Brenner and Guthrie, 2005). The domains are colour coded to correspond to the domains outlined in part A of this figure. C. Ribbon diagram of the structure of yeast elongation factor 2 (EF2) solved by x-ray crystallography. The predicted structure of Snu114p was based on the structure of EF2. Domains I, G', II, III, IV and V of EF2 are labelled on this figure. EF2 struture from Jorgensen et al., 2003.

terminus of Snu114p has been shown to cause synthetic lethality with specific U5 and U6 snRNA mutants, indicating an involvement of the N-terminus of Snu114p in interactions with the U5 and U6 snRNAs (Frazer et al., 2009). The C-terminus of Snu114p also contains unique residues not present in EF-2 (Brenner and Guthrie, 2005). These unique regions are predicted to have a function specific to splicing (Brenner and Guthrie, 2005). Work on hSnu114p has shown the C-terminus of hSnu114p interacts with the C-terminus of another U5 protein, hPrp8p (*Prp8p in S.cerevisiae*) (Liu et al., 2006). Over expression of the C-terminal domain of Snu114p (amino acids 834 to 1008), results in growth inhibition. Further analysis suggests that it is the region between amino acids 834 and 929 that cause the inhibition (Akada et al., 1997). This dominant negative effect may suggest this region has an important function in the interactions or binding activities of Snu114p. Mutagenesis studies have revealed that Snu114p containing a C-terminal deletion is synthetically lethal in combination with Prp28p, Brr2p and Prp8p mutants (Brenner and Guthrie, 2005). As Prp28p, Brr2p and Prp8p are involved in spliceosomal activation, this suggests that the C-terminus of Snu114p plays a role in spliceosomal activation (Brenner and Guthrie, 2005; Kuhn et al., 1999; Raghunathan and Guthrie, 1998; Staley and Guthrie, 1999). It has been proposed that the C-terminus of Snu114p interacts with Prp8p to control or influence Prp28p and Brr2p, which are involved in U1 and U4 snRNP release respectively (Brenner and Guthrie, 2005; Kim and Rossi, 1999; Staley and Guthrie, 1999). Deletion of the C-terminus of Snu114p has also been shown to result in a mutant phenotype at 30^{o}C with release of U4 snRNP being blocked, preventing spliceosomal activation (Brenner and Guthrie, 2006). This retention of U4 snRNP is the same temperature sensitive phenotype observed in Snu114p N-terminal deletion strains, suggesting that unwinding of U4 from U6 snRNA during spliceosomal activation requires both the N- and C-terminal regions of Snu114p (Bartels et al., 2002; Brenner and Guthrie, 2006). Further to

this common function of the N- and C-terminal domains, recent data suggests that both these regions are involved in a common interaction, possibly with Prp8p, and are involved in the addition of the tri-snRNP to the spliceosome (Brenner and Guthrie, 2005). Aside from the possibility that the N- and C-terminal regions share a common function, it has been hypothesised that the C-terminus of Snu114p may act as a domain that communicates conformational changes in Snu114p to other splicing factors (Brenner and Guthrie, 2005). Synthetic lethal studies of Snu114p and the U2, U4, U5 and U6 snRNAs has led to the proposition that the G domain and the N-terminus of Snu114p detect the state of the spliceosome (Frazer et al., 2009). It has also been proposed that the C-terminus of Snu114p communicates the state of the spliceosome, possible via structural rearrangements, to other splicing factors such as Brr2p (Frazer et al., 2009).

1.9.2.2 GTP binding of Snu114p

As Snu114p is the only GTPase known to be involved in splicing, determining the purpose of the G domain of Snu114p has been the focus of much research. Mutations preventing GTP binding in the G domain of Snu114p are lethal, as are several amino acid substitutions in the P-loop of the G domain, indicating that GTP binding is important for the function of Snu114p *in vivo* (Bartels et al., 2003; Dix et al., 1998; Fabrizio et al., 1997). Mutaions in the GTP binding domain of Snu114p, altering its binding affinities to bind XTP rather than GTP, results in a temperature sensitive phenotype, with the U4 snRNA being retained at the restrictive temperature (Bartels et al., 2003). This U4 snRNA retention was still observed after the addition of non-hydrolysable XTP analogues, indicating an importance for Snu114p and its GTP binding and/or hydrolyzing activities in U4/U6 unwinding (Bartels et al., 2003). As mentioned, the same mutant phenotype is observed in both Snu114p N- and C-terminal

deletions, indicating that the GTP binding and/or hydrolyzing activities of Snu114p, as well as the N- and C-terminal domains of Snu114p are important for U4/U6 unwinding (Bartels et al., 2002; Bartels et al., 2003; Brenner and Guthrie, 2006). Mutations in the G domain resulting in temperature sensitive phenotypes have also revealed a role for Snu114p in spliceosome assembly. Growth of cells carrying such mutations, at the restrictive temperature, resulted in decreased levels of U5 and the U4/U6.U5 tri-snRNP, suggesting that GTP binding and/or hydrolysis are important for maintaining appropriate levels of U5 snRNP and the tri-snRNP (Bartels et al., 2003). Snu114p is involved in U4/U6 unwinding, a major step in spliceosomal activation. Recent evidence suggests Snu114p is also involved in the disassembly of the post splicing complex consisting of U2/U6, U5 and the intron. Both U4/U6 unwinding and disassembly of the post spliceosome are repressed by Snu114p bound to GDP and derepressed by Snu114p bound to GTP with out hydrolysis of the GTP, indicating it is GTP binding rather than GTP hydrolysis that is required for these processes (Small et al., 2006). It is possible that this is due to conformational changes in Snu114p as a result of GTP binding (Jorgensen et al., 2003).

1.9.2.3 The relationship between Snu114p and Prp8p

Mutations in Snu114p that disrupt interactions between Snu114p and Prp8p result in defects in U5 snRNP formation and a decrease in Prp8p levels. This suggests that Prp8p stability depends upon its interactions with Snu114p (Brenner and Guthrie, 2006). These mutations also resulted in a decrease in levels of Snu114p and Prp8p bound to U5 snRNA, although the levels of Snu114p in the mutant extract used were similar to those of wild type extracts. This indicates a need for Prp8p in the binding of Snu114p to U5 snRNA (Brenner and Guthrie, 2006). The levels of Brr2p bound to U5 snRNA were also reduced in these mutant extracts,

indicating a close relationship between Snu114p, Prp8p and Brr2p (Brenner and Guthrie, 2006). Disrupting Prp8p binding to Snu114p leads to a decrease in U5 snRNP formation (Bartels et al., 2003). This is also observed when disrupting a predicted interdomain salt bridge within Snu114p, and changing the GTPase domain of Snu114p to an XTPase domain, indicating the GTPase domain and possibly the conformation or folding of Snu114p are also important for U5 snRNP formation (Bartels et al., 2003). Expanding on current knowledge of the relationship between Snu114p and Prp8p, several models of activity have been proposed. The first of these models suggests that Snu114p is in the GTP bound form when the tri-snRNP binds the spliceosome and that GTP hydrolysis is triggered by U1 snRNP. A link between Snu114p and U1 snRNA is also supported by the fact that Snu114p and U1 snRNP proteins Prp39p and Prp40p interact with neighbouring regions of Prp8p (Dix et al., 1998). GTP hydrolysis may lead to conformational changes in Snu114p and Prp8p, and the unwinding of U1/5' splice site duplex and U4/U6 duplex by Prp28p and Brr2p respectively (Brenner and Guthrie, 2005). The second model proposes that GTP hydrolysis is required for stabilising a conformation of Snu114p that is most favourable for Prp8p binding, or that GTP hydrolysis is needed for the rearrangements in activation of the spliceosome and that a stable interaction between Snu114p and Prp8p requires GTP binding, rather than GTP hydrolysis. GTP hydrolysis by Snu114p would occur once the whole spliceosome had formed, allowing the rearrangements required for activation to occur (Brenner and Guthrie, 2006).

1.9.2.4 Posttranscriptional modifications of Snu114p and their potential for regulating Snu114p function

The fact that Snu114p has been shown to be ubiquitinated and Prp8p has been shown to contain the MPN domain which is associated with ubiquitin binding, highlights the possibility

that ubiquitination may affect splicing (Peng et al., 2003; Verma et al., 2002). A role for ubiquitin has also been suggested in repressing U4/U6 unwinding, implementing ubiquitination in timing of events in activation of the spliceosome (Bellare et al., 2008). Further supporting a role for ubiquitin in pre-mRNA splicing, the NTC component Prp19p is thought to exhibit ubiquitin ligase activities (Song et al., 2010). Recent work on human Snu114p (hSnu114p) has revealed that hSnu114p is a substrate for specific phosphatases (Shi et al., 2006). Certain PP1 and PP2A family phosphatases are required for the second step of splicing, with hSnu114p being one of the key substrates (Shi et al., 2006). Based on this data and what is currently known about hSnu114p, it is predicted that dephosphorylation of specific substrates, including hSnu114p, facilitates structural rearrangements in the spliceosome, in the transition between the first and second steps of splicing (Shi et al., 2006). With this in mind one should consider the possibility that phosphorylation and ubiquitination may play a role in the function of Snu114p and in the transition between the first and second step conformations of the spliceosome.

1.9.3 The U5 snRNP protein, Brr2p

Brr2p (also known as Prp44p, Slt22, Rss1p and Snu246p) is an essential U5 snRNP protein, and is the *S.cerevisiae* homologue of the human U5 snRNP protein, U5-200kD or hBrr2p (Lauber et al., 1996). Brr2p, so called due to its Bad Response to Refrigeration, was discovered in a screen of cold sensitive mutants exhibiting defects of pre-mRNA splicing (Noble and Guthrie, 1996). In this screen a *brr2* mutant was found to inhibit splicing before the first step (Noble and Guthrie, 1996). In the same year a Brr2p mutant (*Rss1-1*) was identified as being able to overcome a block in splicing at the 3’ splice site (Lin and Rossi, 1996), and Brr2p was also found in a synthetic lethal screen to identify proteins that are

involved in U2/U6 snRNA interactions (Xu et al., 1996). More recent work has confirmed a role of Brr2p in U4/U6 unwinding during spliceosomal activation and in disassembly of the post splicing complex, probably via U2/U6 unwinding (Raghunathan and Guthrie, 1998; Small et al., 2006).

1.9.3.1 Brr2p is a DExH-box RNA helicase

DEXD/H box ATPase proteins are a family of proteins capable of ATPase and helicase activities. These proteins are classified by the presence of several conserved sequence motifs (Cordin et al., 2005). DEXD/H box ATPase proteins contain a cleft that adopts an open or closed conformation in response to ATP binding. It is thought that ATP hydrolysis results in an open conformation, leading to ADP release and translocation of the protein along several bases of RNA. Binding of ssRNA and ATP causes cleft closing. Then hydrolysis of ATP results in adoption of the open conformation and translocation along RNA. This cycle is thought to be the basis for the helicase activity of the DEXD/H box proteins (de la Cruz et al., 1999).

Sequence analysis of *BRR2* revealed that Brr2p was a putative ATP-dependent RNA helicase, containing two conserved helicase-like domains, present in DExH-box ATPase family members (Lauber et al., 1996; Lin and Rossi, 1996; Noble and Guthrie, 1996; Xu et al., 1996). Several sequence motifs associated with ATP binding and helicase activities were identified within Brr2p (Figure 1.9). The conserved GKT motif is thought to be part of a phosphate-binding loop, or P-loop, required for ATP binding and hydrolysis (Cordin et al., 2006). The DExH motif is required for ATPase and helicase activities, the main role of the SAT motif appears to be helicase activity, and the GRAGR motif is involved in ATPase activity and RNA binding (Cordin et al., 2006). The helicase activity of Brr2p was physically

demonstrated in purified complexes containing Brr2p and in which U4 and U6 are base paired together (Raghunathan and Guthrie, 1998). The addition of ATP to these complexes resulted in disruption of the U4/U6 snRNA base-pairing. However, a *brr2* allele containing a mutation in the first helicase domain of Brr2p did not stimulate this U4/U6 unwinding (Raghunathan and Guthrie, 1998).

1.9.3.2 Structure and domains of Brr2p

Brr2p is an essential, 246 kDa protein, consisting of an N-terminal domain of unknown function, two helicase-like domains (H1 and H2) and two Sec63 domains (S1 and S2) (Figure 1.9). The H1 domain is highly conserved and contains the conserved sequence motifs of the DExH-box RNA helicase family members. The ATPase motifs in the first helicase-like domain are required for cell viability, ATPase activity and pre-mRNA splicing (Kim and Rossi, 1999). The H2 domain is less conserved and the helicase motifs in this region are not essential for cell viability (Kim and Rossi, 1999). The two Sec63 domains are so called due to homology with a protein component of the endoplasmic reticulum translocon complex, Sec63p (Ponting, 2000). The first Sec63 domain is involved in U4/U6 duplex unwinding (Small *et al*., 2006; Pena *et al*., 2009; Zhang *et al*., 2009). The structure of domain S2 of Brr2p has recently been found to resemble domains of a DNA helicase, Hel308 (Pena et al., 2009; Small et al., 2006; Zhang et al., 2009). With the determination of the structure of domain S2 and sequence analysis of Brr2p, it has been proposed that Brr2p is composed of the N-terminal domain, followed by two Hel308-like domains (Pena et al., 2009; Zhang et al., 2009). Similarities of Brr2p with Hel308 and mutational analysis of Brr2p, would suggest that the helicase activities of Brr2p are being carried out in a similar manner to those of Hel308 (Pena et al., 2009; Zhang et al., 2009). Hel308 is thought to use a β-hairpin loop to separate two

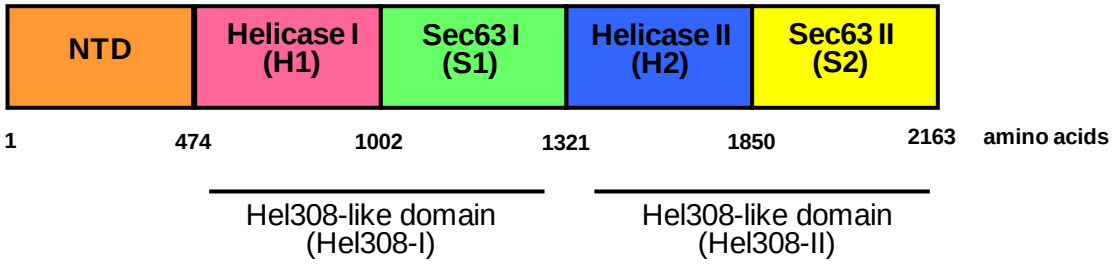


Figure 1.9 The domains of *S.cerevisiae* Brr2p. Brr2p contains an N-terminal domain (NTD), two helicase-like domains (H1 and H2) and the Sec63 domains (S1 and S2). The two helicase-like domains contain several sequence elements indicative of a DExH-box ATPase family member. These sequence elements are displayed below the schematic of Brr2p. Brr2p is thought to contain two regions similar to domains present in the DNA helicase Hel308. These Hel308-like domains are named Hel308-I, which consists of domains H1 and S1, and Hel308-II, which consists of domains H2 and S2 of Brr2p. These are labelled as Hel308-I and Hel308-II.

strands of DNA, and uses a ratchet helix that relies on ATP binding and hydrolysis for nucleic acid translocation (Buttner et al., 2007). If this were the case, Brr2p would be far more processive than other DExH-box proteins.

1.9.3.3 Functions of Brr2p

Initial studies of Brr2p demonstrated a role for this protein prior to the first step of splicing, with a *brr2* mutant identified that blocked splicing before the first step at low temperatures and a mutant able to overcome a block to splicing introduced at the 3' splice site (Lin and Rossi, 1996; Noble and Guthrie, 1996; Xu et al., 1996). It was also revealed that the ATPase activities of Brr2p are linked to annealing of U2/U6 snRNAs (Xu et al., 1996). Genetic interactions have been identified between *BRR2* and the U2, U4, U5 and U6 snRNAs, and interaction of Brr2p with Prp8p, Prp16p, Snp1p, Slu7 and Snu66p have also been identified (Kuhn and Brow, 2000; van Nues and Beggs, 2001; Xu et al., 1998; Xu et al., 1996). This gives a clear indication that Brr2p is an important spliceosome component, located centrally within the spliceosome and led to the proposition that Brr2p is involved in the recruitment of Prp16p, which is required for the second step of splicing (Kuhn and Brow, 2000; van Nues and Beggs, 2001; Xu et al., 1998; Xu et al., 1996). Investigations into the protein-protein interactions of Brr2p also revealed that the C-terminal half of Brr2p is the major site of protein interactions (van Nues and Beggs, 2001). Following Brr2p being identified as the homologue of the human U5 snRNP protein, U5-200kD (hBrr2p), the role of Brr2p in pre-mRNA splicing was further defined, with a role in U4/U6 unwinding during activation of the spliceosome being revealed (Laggerbauer et al., 1998). First hBrr2p was shown to mediate ATP-dependent unwinding of the U4/U6 RNA duplex *in vitro* (Laggerbauer et al., 1998). Experiments in yeast followed, to demonstrate that Brr2p and ATP hydrolysis were required for unwinding of the

U4/U6 snRNA duplex in yeast (Raghunathan and Guthrie, 1998). Further studies have shown that it is motifs in the first helicase-like domain (H1) that are required for U4/U6 duplex unwinding *in vivo* (Kim and Rossi, 1999). It has also been shown that the C-terminus of Prp8p is required for the ATP dependent U4/U6 unwinding activities of Brr2p (Maeder et al., 2009). With the identification of *brr2* mutants *brr2*-E610G and *brr2*-R1107A, which prevented the release of excised introns, Brr2p was also implicated in disassembly of the post splicing complex (Noble and Guthrie, 1996; Small et al., 2006).

Brr2p is present in the tri-snRNP and persists in the spliceosome throughout the whole of the splicing cycle (Lauber et al., 1996). However, the activities of Brr2p are only thought to be needed for unwinding of the U4/U6 duplex during activation of the spliceosome, and at disassembly of the post splicing complex, probably for U2/U6 unwinding, so the activities of Brr2p need to be tightly regulated. One proposed source of control is the GTP state of Snu114p (Small et al., 2006). Snu114p is also known to be involved in both U4/U6 unwinding and disassembly of the post splicing complex, indicating a common pathway for activation and disassembly of the post splicing complex, involving Brr2p and Snu114p (Small et al., 2006). It has been shown that U4/U6 unwinding and disassembly of the post splicing complex are repressed by GDP bound Snu114p and derepressed by GTP bound Snu114p (Small et al., 2006). Therefore it is thought that GTP bound Snu114p promotes Brr2p activity in spliceosomal activation and disassembly of the post-splicing complex, and GDP bound Snu114p represses the activities of Brr2p when not required (Small et al., 2006). This has led to the theory that Snu114p is acting as a signal dependent switch rather than a molecular motor, communicating with Brr2p to control its activities (Small et al., 2006). Prp8p is also thought to play a role in the regulation of Brr2p. Extensive interactions have been identified

between Brr2p and Prp8p, and the C-terminus of Prp8p has been shown to be required for U4/U6 duplex unwinding by Brr2p, possibly by facilitating the binding of Brr2p to the U4/U6 duplex (Kuhn and Brow, 2000; Maeder et al., 2009; van Nues and Beggs, 2001; Zhang et al., 2009).

1.9.3.4 Brr2p and Retinitis Pigmentosa

Two mutations in the human homologue of Brr2p have been linked to the degenerative eye disease, autosomal dominant Retinitis Pigmentosa (Li et al., 2010; Zhao et al., 2009). The yeast equivalents of these mutations in Brr2p, N1104L and R1107L, are both situated in the Hel308-like ratchet helix, in the S1 domain, thought to be required for nucleic acid translocation (Zhang et al., 2009; Zhao et al., 2009). Both yeast mutations result in defects in U4/U6 duplex unwinding, suggesting U4/U6 unwinding could be a molecular basis for autosomal dominant Retinitis Pigmentosa (Zhao et al., 2009). Mutations in the C-terminus of Prp8p, which disrupt the interaction of Prp8p with Brr2p, have also been linked to Retinitis Pigmentosa in humans (Boon et al., 2007; Pena et al., 2007). As the C-terminus of Prp8p is required for the U4/U6 unwinding activities of Brr2p, this further supports U4/U6 as a molecular basis for autosomal dominant Retinitis Pigmentosa (Maeder et al., 2009).

1.9.4 Physical and functional links between Brr2p, Snu114p and Prp8p

The human homologues of Brr2p, Snu114p and Prp8p, along with a 40 kDa protein, have been shown to form a stable RNA free complex (Achsel et al., 1998). There are extensive interactions between Brr2p, Snu114p and Prp8p. Work on the human homologues of Prp8p, Snu114p and Brr2p (hPrp8p, hSnu114p and hBrr2p) has shown that the N- and C-terminal fragments of hPrp8p (amino acids 1-387 and 1986-2235, respectively) interact with hSnu114p

between amino acids 279 and 692, and with hBrr2p between amino acids 1301 and 1861 (Liu et al., 2006). The C-terminal region of hPrp8p (amino acids 1986-2235) interacts with the C-terminal region of hSnu114p, between amino acids 603 and 972 (Liu et al., 2006). The region of hSnu114p between amino acids 279 and 972 also interacts with hBrr2p between amino acids 1301 and 1816 (Liu et al., 2006). In yeast the C-terminal half of Brr2p, the second Hel308 domain, interacts with N- and C-terminal regions of Prp8p (amino acids 223-518 and 1822-2395, respectively), and with Snu114p (Zhang et al., 2009). Deletion of the second Sec63 domain of Brr2p reduces binding of Prp8p and Snu114p (Zhang et al., 2009). Snu114p interacts with Prp8p between amino acids 437 and 770 of Prp8p (Boon et al., 2006). The region of Prp8p between amino acids 420 and 542 has also been shown to interact with Snu114p, between amino acids 136 and 511 (Grainger et al., 2009).

A mutation in the MPN domain of Prp8p has been shown to reduce interactions with Brr2p (Liu et al., 2006; van Nues and Beggs, 2001). Mutations effecting the interactions between Snu114p and Pr8p have been shown to result in a decrease in associations of Prp8p, Snu114p and Brr2p with the U5 snRNA, further supporting the close relationship between Snu114p, Prp8p and Brr2p (Brenner and Guthrie, 2006). A yeast two-hybrid screen has identified an interaction between Brr2p and the C-terminus of Prp8p and between the N-terminal region of Prp8p and the C-terminal region of Brr2p (van Nues and Beggs, 2001). Genetic interactions have also been found between the C-terminus of Snu114p and Brr2p (Brenner and Guthrie, 2005, 2006). The C-terminal region of Prp8p (amino acids 1822 to 2395) aids the binding of the C-terminus of Prp8p and Brr2p to the U4/U6 duplex (Zhang et al., 2009). A separate study has demonstrated that amino acids 1806 to 2413 of Prp8p are required for the helicase activity of Brr2p during U4/U6 duplex unwinding (Maeder et al., 2009). It is possible that the increase seen in helicase activity of Brr2p in the presence of the

C-terminal fragment of Prp8p is due to an increase in the interaction between Prp8p and Brr2p with the U4/U6 duplex (Zhang et al., 2009). Electron microscopy studies on Prp8p, Snu114p and Brr2p with in the yeast spliceosome have shown that the U5 snRNA is present in a central body domain of the tri-snRNP, the U4/U6 snRNPs are located in an arm domain and there is a head domain adjacent to the arm domain (Hacker et al., 2008). Brr2p was shown to be located in this head domain, whereas Snu114p and Prp8p were both shown to be located in the body domain (Hacker et al., 2008).

As well as there being extensive interactions between Brr2p, Snu114p and Prp8p, they also appear to function together, with all three proteins being involved in U4/U6 duplex unwinding during spliceosome activation, and disassembly of the post splicing complex. As outlined above, the GTP state of Snu14p is thought to control the activities of Brr2p during U4/U6 duplex unwinding and disassembly of the post splicing complex (Small et al., 2006). As it is known that the C-terminal region of Prp8p is required for the helicase activity of Brr2p (Maeder et al., 2009), and given the extensive interactions between Prp8p, Snu114p and Brr2p, it has been proposed that the GTP state of Snu114p controls Brr2p indirectly via Prp8p (Maeder et al., 2009). In this model the GTP state of Snu114p would effect the conformation of Prp8p, modulating the availability of the C-terminus of Prp8p to interact with Brr2p (Maeder et al., 2009). It has also been proposed that the G domain of Snu114p senses the state of the U4/U6 helix. It is thought that Snu114p then communicates the state of the U4/U6 helix, via conformational changes and possibly via Prp8p, signalling when U4/U6 unwinding is necessary (Frazer et al., 2009).

1.10 The active site of the spliceosome:- The current model

As group II introns, which are self splicing, undergo splicing with identical chemistry to pre-mRNA splicing, it was thought that the spliceosome was a ribozyme (Collins and Guthrie, 2000). However, evidence is increasing to support the theory that the spliceosome is actually an RNP enzyme. In light of mutagenesis data and recent structural studies regarding Prp8p, it has been proposed that the RNase H like domain of Prp8p forms the active site of the spliceosome (Abelson, 2008). Mutations in this domain suppress mutations in the U4 snRNA, 5' splice site, 3' splice site and polypyrimidine tract, and amino acids within the RNase domain are known to crosslink to the 5' splice site (Collins and Guthrie, 1999; Query and Konarska, 2004; Reyes et al., 1999; Umen and Guthrie, 1996). This has led to a model in which, as well as holding both exons at the core of the spliceosome, Prp8p is also involved in the metal-ion chemistry occurring there (Abelson, 2008; Pena et al., 2008). It is thought that the whole process of spliceosome assembly, activation and catalysis occurs while the pre-mRNA is bound to this RNase H like domain of Prp8p (Abelson, 2008).

1.11 Aims

The U5-snRNP proteins Brr2p, Snu114p and Prp8p are essential for pre-mRNA splicing, specifically for unwinding of U4/U6 snRNAs and disassembly of the post splicing complex.

The sequence similarities between Snu114p and EF-2 have been the focus of much of the Snu114p research. It is predicted that the function of Snu114p may be similar to that of EF-2 within the ribosome. Another area of interest arising from this homology is the function or purpose of the residues or regions of Snu114p that are unique and absent in EF-2, and defining the functions of these unique regions, and the domains containing them. One aim of this study was to tag, express and purify specific regions of Snu114p that are thought to have important

functions in splicing, including the unique N-terminal domain, the C-terminal domain and the N-terminus plus the G domain. Obtaining purified fragments of Snu114p would allow structural and functional analysis of specific domains of Snu114p in attempts to define the function of each domain of the protein, and determine the function of the amino acids that are present in Snu114p and absent in the close relative EF-2. This would also start the process of solving the structure of Snu114p in a domain by domain manner, similar to that currently being undertaken to solve the structure of Prp8p.

The second aim of this study was to investigate the requirements for Brr2p, Snu114p and Prp8p association with the U5 snRNA. Although it is well documented that Brr2p, Snu114p and Prp8p are all part of the U5 snRNP, little is known about the assembly of the U5 snRNP and the associations of the proteins with the snRNA especially in yeast. U5 snRNA mutants were constructed to investigate the effects of these mutations on associations of Brr2p, Snu114p and Prp8 with the U5 snRNA. This work will define the regions of U5 snRNA that are necessary for association of Brr2p, Snu114p and Prp8p and determine if Brr2p, Snu114p and Prp8p contact the U5 snRNA as a complex or independently of each other. This will further increase knowledge and understanding of the protein-RNA interactions that occur within a major component at the catalytic core of the spliceosome.

2 Materials and Methods

2.1 Yeast and bacterial strains

All bacteria strains used in this study are listed in Table 2.1, and all yeast strains used are listed in Table 2.2.

2.2 Oligonucleotides

All oligonucleotides used in this study, and their sequences, are listed in Table 2.3.

2.3 Plasmids

Details of all plasmids used in this study are listed in Table 2.4.

2.4 Bacterial Media

2.4.1 LB

Luria-Bertani (LB) media contained 10 mg/ml bacto-tryptone, 5 mg/ml bacto-yeast extract and 10 mg/ml NaCl. LB plates were made with the addition of 2% (w/v) Agar. Antibiotic selection was achieved with the addition of 100 µg/ml carbenicillin, 50 µg/ml kanamycin, or 50 µg/ml chloraphenicol.

2.4.2 Overnight express

Overnight express, auto induction media contained 60 g/l Overnight Express (Novagen) and 10 ml/l glycerol. This media enables auto-induction of T7 promoters, via the utilisation of different carbon sources including glycerol and lactose to control the lac operon.

2.4.3 PASM-5052

PASM-5052 media was made according to Studier, 2005, containing 50 mM sodium hydrogen phosphate, 50 mM potassium hydrogen phosphate, 25 mM ammonium sulphate, 2 mM magnesium sulphate, 200 mg/l each amino acid (apart from cysteine and tyrosine which were omitted), 0.5% glycerol, 0.05% glucose and 0.2% lactose. For ^{15}N labelling ammonium sulphate was omitted, and 1g/l $^{15}NH_4Cl$ (ammonium chloride, Sigma) was added.

2.5 Yeast media

2.5.1 YPD

Yeast extract/peptone/dextrose (YPD) media contained 1% bacto-yeast extract, 2% bacto-peptone and 2% D-glucose. YPD plates were made with the addition of 2% (w/v) agar.

2.5.2 SD drop out media

Synthetic defined (SD) media contained 27 mg/ml SD media drop out base (1.7 mg/ml yeast nitrogen base, 20 mg/ml dextrose, 5 mg/ml ammonium sulphate) (Q-BIOgene) and an amount of complete supplement (for auxotrophic selection of yeast) as instructed by manufacturer (Q-BIOgene). For SD drop out plates 2% (w/v) agar was added.

2.5.3 5FOA plates

2 mg/ml 5FOA (Melford) was dissolved in media containing 4 mg/ml yeast nitrogen base, 0.1 mg/ml of uracil and 0.1 mg/ml of each amino acids required for growth, and 4% (w/v) glucose. This was added to an equal volume of autoclaved 4% (w/v) agar, mixed and poured into plates.

All media was autoclaved at 121°C for 15 minutes.

2.6 Alkaline phosphatase treatment of DNA

Prior to ligations, digested plasmid vectors were alkaline phosphatase treated with calf intestinal phosphatases (CIP, NEB) to prevent religation. 5 units of CIP per µg of DNA were added to restriction enzyme digested plasmid. This was followed by incubation at 37°C for 1 hour, and purification via GenElute PCR purification kit (Sigma) according to the manufacturers instructions.

2.7 Transformation of competent XL1 Blue MRF' bacteria cells

Chemically competent (Hanahan et al., 1991) bacterial cells were defrosted on ice. For the transformations of ligations, 80 µl of cells were added to each 20 µl ligation reaction. For transformation of other plasmids 0.1 µg of DNA was added to 20 µl competent cells. Cells were incubated on ice for 30 minutes, then heat shocked at 42°C for 2 minutes. 100µl of LB was added and cells were incubated at 37°C for 1 hour. Cells were plated on to LB plates containing appropriate antibiotics and incubated at 37°C overnight.

2.8 Extraction and purification of plasmid DNA from XL1Blue-MRF' bacterial cells

A single bacterial colony was grown in 2 ml LB + appropriate antibiotic at 37°C overnight. The cells were spun at 15600 g for 1 minute and plasmid DNA was extracted and purified using the GenElute HP plasmid miniprep kit (Sigma) following the manufacturers instructions.

2.9 DNA Sequencing by MWG

2 µg plasmid DNA was precipitated with 0.2 volumes of 5 M ammonium acetate, and 2.5 volumes of 95% ethanol, at room temperature overnight. DNA was then spun at 15600 g for 5 minutes and washed in 100% ethanol. Dried DNA pellets were sent to MWG for sequencing. Or 20µl plasmid mini prep was sent to MWG for sequencing. All expression constructs and products of mutagenesis were confirmed by sequencing.

2.10 Production of Single Stranded DNA for Oligomutagenesis

BW313 (dut^-, ung^-) bacterial cells were transformed with plasmid to be mutated, using the standard bacterial transformation protocol described in section 2.7. Single colonies were grown in 1 ml LB media containing 100 µg carbanicillin and 2 µg uridine, at 37°C with shaking. After 2 hours, 1 µl VCSM13 helper phage (Stratagene) was added to each culture, and after a further 2 hours 70 µg kanamycin was added. Cells were incubated at 37°C with shaking for 16-24 hours. Cells were harvested by centrifugation at 15600 g for 5 minutes. 800 µl of supernatant was transferred to a new tube and 0.25 volume of 20% PEG/2.5 M sodium chloride was added, and incubated on ice for 30 minutes. Samples were centrifuged at 15600 g for 5 minutes and supernatant was discarded. Pellets were resuspended in 120 µl 0.3 M sodium acetate pH 5.3, 1 mM EDTA. 1 volume of TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) buffered Phenol:Chloroform:isoamyl (125:24:1) alcohol was added, samples were vortexed and spun for 2 minutes at 15600 g. The aqueous phase was transferred to a new tube and 2.5 volumes of 100% ethanol were added and samples were precipitated at -20°C for 30 minutes. Samples were spun at 15600 g for 5 minutes and pellets were washed in 96% ethanol. Pellets were air dried and resuspended in 10 µl TE buffer.

2.11 Oligomutagenesis using T4 DNA polymerase

40 ng of a mutagenesis primer was 5'-phosporylated in a 20 µl reaction containing 1X Ligase buffer (Roche) and 5 units T4 polynucleotide kinase (T4 PNK, NEB). Reactions were incubated at 37°C for 30 minutes, then at 65°C for 20 minutes. Primers were hybridized to single stranded DNA (ssDNA) via the addition of 3 µl ssDNA and 1.3 µl 20X SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7), and heating to 70°C, then cooling to 30°C. 20 µl of this hybridization mix was used to set up 100 µl extension reactions containing 20 mM HEPES pH 7.8, 2 mM DTT, 10 mM magnesium chloride, 0.5mM each dNTP, 1mM ATP, 1 unit T4 DNA polymerase (Roche), 0.5 units T4 DNA ligase (Roche). Extension reactions were incubated on ice for 5 minutes, room temperature for 5 minutes, then at 37°C for 3 hours and finally put on ice. 4 µl of each extension reaction was transformed into competent XL1 Blue MRF' cells using the standard bacterial transformation protocol. Plasmid DNA was isolated from the resulting colonies and sequenced to identify plasmids containing the correct mutation.

2.12 Yeast transformation

Yeast transformations were carried out according to the lithium acetate method (Gietz et al., 1992). A single colony was grown overnight in 5 ml YPD at 30°C with shaking. This culture was used to set up a 25 ml culture in YPD at OD_{600} of 0.5. This culture was grown at 30°C with shaking for around 4 hours to reach on OD_{600} of 2. Cells were pelleted by centrifugation at 2000 g for 5 minutes, washed in water and resuspended in 700 µl 0.1M lithium acetate. Cells were pelleted by centrifugation at 15600 g for 1 second and resuspended in 400 µl 0.1M lithium acetate. 50 µl cells were aliquoted for each transformation, spun at 15600 g for 1 second and lithium acetate was removed. A transformation mix containing 240 µl 50% PEG

3500, 36 µl 1M lithium acetate, 50 µl 2 mg/ml salmon sperm DNA (boiled for 5 minutes) and 1 µg of each plasmid to be transformed, was added to each cell pellet. Reactions were made up to 360 µl with water, vortexed, then incubated at 30°C for 30 minutes, and 42°C for 30 minutes. Cells were pelleted by centrifugation at 4500 g for 1 second. Transformation mix was removed and pellets were resuspended in 1 ml water. 100 µl of this was plated onto an appropriate SD plate and incubated at 30°C for 3 days.

2.13 Production of whole cell yeast extract by liquid nitrogen breakage

Yeast whole cell extracts were produced according to Ansari and Shwer, 1995 (Ansari and Schwer, 1995). Yeast cells were grown in appropriate media at 30ºC to an OD_{600} of between 2 and 4. Cells were spun at 3000 rpm for 5 minutes in a Sorvall evolution with SLC3000 rotor, washed in cold water, then washed in AGK buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM magnesium chloride, 200 mM potassium chloride, 0.5 mM DTT, 10% glycerol). For extracts used in Prp8p antibody immunoprecipitations pellets were resuspended in 1 ml AGK buffer, frozen and lysed in an SPEX Sample Prep, 6770 Freezer/Mill. A further 2.75 ml AGK buffer was then added. For all other extracts pellets were resuspended in 3.75 ml of AGK buffer. Cells were frozen and ground to fine powder, lysing the cells, under liquid nitrogen. Cell paste was defrosted on ice and stirred at 4ºC for 30 minutes. This was then spun at 17000 rpm for 30 minutes in a Beckman J25 with a JA25.50 rotor. Supernatant was spun at 40000 rpm for 1 hour in a Beckman L-90K ultra centrifuge with a Ti70.1 rotor. The upper 2/3 of supernatant was removed and dialysed for 3 hours in buffer D (20 mM HEPES-KOH pH 7.9, 0.2 mM EDTA, 50 mM potassium chloride, 0.5 mM DTT, 20% glycerol). Dialysed extract was spun at 17000 rpm for 10 minutes in a Beckman J25 with a JA25.50 rotor. Extract was aliquoted, frozen in liquid nitrogen and stored at -80ºC.

2.14 Production of bacterial constructs to express fragments of Snu114p

2.14.1 Amplification of *SNU114* fragments via PCR

PCR reactions were carried out using 1 µM of each dNTP (Amersham), 1X Accubuffer (Bioline), 1 µM of each primer (see Table 2.3 for primer details), 2 mM $MgCl_2$ (Bioline), 10 ng pRS413-Snu114 plasmid template, and 1.25 units of Accuzyme (Bioline) in 50 µl reactions. The thermal cycling parameters used for the reactions were:- 1 cycle of 95°C for 5 minutes, 35 cycles of 1 minute at 95°C, 1 minute at 54°C and 3 minutes at 72°C, followed by 1 cycle of 10 minutes at 72°C. PCR products were analysed on 0.8% agarose gel containing ethidium bromide (0.5 ng/ml), along with 1kb standard (NEB). Four PCR products were pooled and purified using the GenElute PCR purification kit (Sigma) according to the manufacturers instructions.

2.14.2 Ligation of *SNU114* fragments into pBluescript II KS+

2 µg pBluescript II KS+ (pBSIIKS+), and four 50 µl pooled and purified PCR reactions of amplified *SNU114* fragments were digested with EcoRI (Roche). EcoRI restriction digests were carried out in 50 µl reactions containing 1X buffer H (Roche), 1X BSA (NEB), 20 units of EcoRI (Roche). Reactions were incubated at 37°C for 1 hour. DNA was purified using GenElute PCR purification kit (Sigma) following the manufactures protocol. Purified DNA was then digested with EagI in 50 µl reactions with 1X buffer 3 (NEB), 1X BSA (NEB), and 20 units EagI (NEB). Reactions were incubated overnight at 37°C and DNA was purified using GenElute PCR purification kit (Sigma) following the manufactures protocol.

Digested, purified *SNU114* fragments were ligated into digested, alkaline phosphatase treated and purified pBSIIKS+ to allow sequencing of the amplified regions of *SNU114*. Ligations were set up in 20 µl reactions containing 1X ligation buffer (Roche), 1 unit T4 DNA ligase

(Roche), a 4 to 1 ratio of insert to vector. Reactions were incubated at room temperature for 90 minutes and transformed into competent XL1Blue-MRF' cells and subjected to blue-white colour selection.

2.14.3 Blue-white colour selection

For blue-white colour selection of pBIIKS+ containing cells were plated out onto LB+ Carbenicillin (Carb, 100 µg/ml, Duchera) agar plates, previously spread with 100 µl X-Gal (20 mg/ml) and 100 µl 40 mM IPTG. Plates were incubated at 37°C overnight. Colonies expected to contain the insert will appear white.

2.14.4 Ligation of *SNU114* fragments into pET24b and pET28a

SNU114 fragments were ligated into pET28a and pET24b vectors (Novagen) to allow expression in bacteria and achieve both N- and C-terminally tagged protein fragments, respectively.

pBSIIKS+ plasmids containing a *SNU114* fragment with the correct sequence (confirmed by sequencing by MWG), and pET24b and pET28a vectors (2 µg) were digested, with EagI in 50 µl reactions containing 1X buffer 3 (NEB), 1X BSA (NEB), and 20 units EagI (NEB). Reactions were incubated overnight at 37°C and DNA was purified using GenElute PCR purification kit (Sigma) following the manufactures protocol. This was followed by digestion with NdeI (NEB) in 50 µl reactions containing 1X buffer 4 (NEB), 1X BSA (NEB) and 20 units NdeI (NEB). Reactions were incubated at 37°C for 90 minutes. Digested pET vectors were alkaline phosphatase treated and purified using the GenElute PCR purification kit (Sigma) according to the manufacturers protocol. Excised *SNU114* fragments were gel purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturer

instructions. Excised fragments were ligated into pET24b and pET28a in 20 µl reactions containing 1X ligation buffer (Roche), 1 unit T4 DNA ligase (Roche), and a 4 to 1 ratio of insert to vector. Reactions were incubated at room temperature for 90 minutes and transformed into competent XL1Blue-MRF' cells and plated out onto LB+ Kanamycin (Kan, 50 µg/ml, Melford) agar plates. Plasmids were isolated and test digested. Vectors containing the *SNU114* fragment were transformed into competent BL21 (DE3) and Rosetta 2 (DE3) cells.

2.14.5 Transformation of expression constructs into BL21 (DE3) bacterial cells (Novagen)

Competent bacterial (Hanahan et al., 1991) cells were defrosted on ice, then 1µl of DNA (0.1-0.2 µg/µl) was added to 80 µl of competent cells. The transformation procedure was carried out as described for XL1 Blue MRF' cells.

2.14.6 Transformation of Rosetta 2 (DE3) bacterial cells (Novagen) with expression constructs

Cells were thawed on ice. 1 µl of plasmid DNA (0.1-0.2 µg/µl) was added to 20µl of Rosetta 2 (DE3) cells (Novagen) and were mixed gently, then incubated on ice for 5 minutes. Cells were heat shocked at 42°C for 30 seconds, and incubated on ice for 2 minutes. 80 µl of SOC media (Novagen) was added to the cells, followed by 1 hour incubation at 37°C with shaking. Cells were plated out on appropriated LB agar plates, which must contain 50 µg/ml Chloramphenicol (Chlor, Sigma), to select for the pRARE2 plasmid present in Rosetta2 cells (pRARE2 plasmid expresses seven rare tRNAs), along with 50 µg/ml Kan to select for the pET 28a and 24b vectors. Plates were incubated at 37°C overnight.

2.15 Expression and purification of recombinant Snu114p fragments

2.15.1 Small scale test expression of Snu114p fragments

Small scale expression and purifications were carried on each bacterial strain to test for the combination of vector and bacterial cells resulting in the highest expression of soluble protein fragment. The vector and bacterial cells giving highest expression were used for large scale expression. A 2 ml LB+ Kan (50 μg/ml) preculture was grown at 30 and 37°C was added to 30 ml overnight express (Novagen) + Kan (50 μg/ml). This 30 ml culture was incubated overnight at the same temperature as the preculture. Cells were spun at 12000 rpm for 5 minutes, at 4°C in a Beckman J-25 centrifuge with JA25.50 rotor. Pellets were resuspended in 7.5 ml of lysis buffer (50 mM sodium phosphate (pH 8), 300 mM sodium chloride, 10 mM imidazole). Resuspended pellets were sonicated (Vibra cell, Jencons) with four bursts of 20 seconds with a tapered microtip at an amplitude of 30%, to lyse the cells. Lysed cells were spun at 17000 rpm for 15 minutes at 4°C, in a Beckman J-25 centrifuge with JA25.50 rotor, pelleting the cell debris. The resulting lysate from this spin was applied to a column for purification.

2.15.2 Small scale column purification of His-tagged Snu114p fragments

For small scale purification a 10 ml column was loaded with 0.5 ml HIS-Select Nickel affinity beads (Sigma). Beads were washed with wash buffer (50 mM sodium phosphate (pH 8), 300 mM sodium chloride, 20 mM imidazole). Extract was then applied to the column and allowed to flow through. 1ml of the flow through was collected for gel analysis. The beads were then washed twice with wash buffer, with 1 ml of the flow through being collected for gel analysis. Proteins were eluted in fractions with elution buffer (50 mM sodium phosphate (pH 8), 300 mM sodium chloride, 250 mM imidazole). A small amount of pellet from the extraction was

resuspened in SDS loading buffer for gel analysis. 5 µl of collected fractions and samples were analysed on 12% SDS-PAGE gels, with 5 µl water and 10 µl SDS loading buffer (60 mM Tris-Cl, pH6.8, 10% glycerol, 2%SDS, 5% 2-mercaptoethanol, 0.5% bromophenol blue). All purification steps were carried out at 4°C.

2.15.3 Large scale expression of Snu114p fragments

Five 2 ml pre-cultures of bacterial expression strains in LB+ Kan (50 µg/ml) were grown at 37°C overnight. These were added to 1l of PASM-5052 (Studier, 2005) or overnight express + Kan (50µg/ml), which was grown at 37°C for 38 to 40 hours. Cells were spun at 12000 rpm for 5 minutes, at 4°C in a Beckman J25, JA14. Pellets were resuspended in 7.5 ml of lysis buffer per 250 ml culture spun down. Cells were lysed as outlined for small scale expressions.

2.15.4 Large scale column purification of His tagged Snu114p fragments

For large scale purification a 25 ml column (Biorad) was loaded with 1.5 ml of HIS-Select Nickel affinity beads (Sigma). Purification was carried out as outlined for small scale purifications. Purified protein was dialysed and protein concentrations were measured using the BCA kit (Pierce).

2.15.5 Coomassie Blue Staining

SDS-PAGE gels were stained in Coomassie Blue (0.1% Coomassie blue in 40% methanol, 10% acetic acid) for 30 minutes, and destained for 2 hours in gel destain (40% methanol, 10% acetic acid).

2.15.6 Protein concentration measurement via BCA

All protein concentrations were measured using the BCA™ Protein Assay Kit (Pierce). 1ml of working reagent was added to 50 µl of protein sample to be measured. This was incubated at 37°C for 30 minutes then incubated at room temperature for 30 minutes. The absorbance at 562 nm was measured.

2.15.7 Concentrating dialysed protein

When protein concentrations higher than that of the dialysed protein were required, dialysed proteins were concentrated in spin column (mwco=5000 kDa, VivaScience), by spinning at 4,000 rpm for 20 to 30 minutes in a Sorvall Legend RT.

2.16 Western blotting

Protein samples were added to 1 volumes of SDS loading dye, were heated at 90°C for 5 minutes, then separated on a 12% SDS-PAGE gel with 10 µl Precision Plus Protein™ Dual colour standard (BioRad). The SDS-PAGE gel was placed on nitocellulose membrane (Schleicher & Schuell, 0.45 M pore), between blotting paper (extra thick, PROTEAN®xi size, BioRad), all of which were soaked in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol, 1% SDS). The semi-dry electrotransfer ran at 10 V for 1 hour in a semi-dry transfer cell (Biorad, Trans-blot SD). The membrane was rinsed in TBS (50 mM Tris pH 0, 150 mM sodium chloride), blocked in blocking buffer (1X TBS, 0.05% Tween-20, 5% (w/v) dry milk powder (Marvel)), incubated in blocking buffer with primary antibody for 1 hour and washed three times with blocking buffer. The membrane was incubated for 1 hour in blocking buffer and secondary antibody, then washed twice in blocking buffer, and three times in TBS-T (1X TBS, 0.05% Tween-20). Membrane was incubated with 1 ml Luminol/Enhancer solution and

1 ml Stable Peroxide Solution (Super Signal® West Dura Extended Duration Substrate, Pierce). Western blots were exposed for an appropriate time to x-ray film (Fuji). Details of the antibodies used are outlined in Table 2.5.

2.17 Mass spec analysis

Protein was dialysed in 10mM Tris-HCl, pH 7.4, 150 mM NaCl and concentrated to 50 pmol/µl. Mass spec analysis was carried out by the Biomolecular Analysis Facility in the Michael Smith Building, The University of Manchester.

2.18 X-ray crystalography

2.18.1 Crystal growth trails with Snu114p N-terminus

Small scale, sitting drop, crystal growth trials were set up in 96 well intelliplates (Hampton Research) using a Pheonix robot (Art Robbins Instruments). Trials were set up with four different screens of crystalisation conditions:- the Classic Suite, SM1 Suite, PEGs Suite and JCSG+ Suite (Qiagen). 0.1 µl of well solution and 0.1 µl of the purified N-terminus of Snu114p dialysed in buffer D (15 mg/ml), were mixed in the small wells of 96 well plates. Plates were sealed and stored at room temperature.

2.18.2 X-ray diffraction

Crystals were frozen in a nitrogen vapour stream. X-ray diffraction was carried out carried out with a detector (RAXIS-IV) to crystal distance (D) of 150. Diffraction was detected with crystals turning 0.5° with a 3 minutes exposure. Images were captured in Rigaku, Crystal clear, version 1.3.6.

2.19 NMR

Unlabeled and labeled protein was dialysed in 50 mM sodium phosphate, pH 6.2, 100 mM sodium chloride, concentrated to 10 mg/ml. NMR analysis was carried out using Bruker 600 and 700 MHz NMR spectrometers at The National Institute of Medical Research, Mill Hill, London. The experiments were performed by John King and Tony Cheung from the lab of Dr V Ramesh at The University of Manchester.

2.20 *In vitro* pre-mRNA splicing to investigate the effects of addition of Snu114p N-terminus

2.20.1 Labelling of actin pre-mRNA splicing substrate

Plasmid containing actin (p283) was linearised with BamH1 (NEB). 10 µl run-off transcription reactions were set up containing 1X T7 transcription buffer (40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 1X nucleotides (0.5 mM ATP, CTP and UTP, 25 µM GTP), 10 mM DTT, 1 µg digested p283, 50 µCi 400Ci/mmol α^{32}P GTP (Amersham), 20 units RNAsin (Promega) and 20 units T7 polymerase (Promega). Reactions were incubated at 37ºC for 30 minutes. 20 µg of tRNA, 0.5 µl 0.5 M EDTA, 100 µl water and 100 µl of phenol (pH 4.3) were added to reactions. These were vortexed for 2 minutes and spun at 15600 g for 2 minutes. The aqueous phase was transferred to a new tube and 0.2 volumes of 5 M ammonium acetate and 3 volumes of 100% ethanol were added and RNA was precipitated at -20ºC for at least 30 minutes. RNA was spun at 15600 g for 5 minutes. The RNA pellet was resuspended in 6 µl of Formamide loading buffer, boiled for 1 minute and ran on a 6% acrylamide/8 M urea gel, for 90 minutes at 22 W. The gel was exposed to film (Fuji) to locate the band of labelled actin pre-mRNA. The band was excised from the gel and the RNA was extracted via electroeluting at 200 V for 1 hour in a Centrilutor, micro-electoelutor (Amicon), in 1X TBE,

1% SDS. The resulting solution in a centricon filter tube (YM-30, Millipore) was spun at 5600 g for 40 minutes, then the column was turned over and the remaining buffer containing the RNA was spun at 1870 g for 2 minutes. The volume of collected buffer containing RNA was made up to 100 µl with H_2O and an equal volume of citrate buffered phenol was added. This was vortexed for 2 minutes, spun at 15600 g for 2 minutes The aqueous phase was transferred to a new tube. 0.1 volumes of 3 M sodium acetate (pH 5.3) and 2.5 volumes of 100% ethanol were added to the aqueous phase and precipitated at -20^{o}C for at least 30 minutes. The RNA was spun down at 15600 g for 5 minutes and resuspended in an appropriate volume of water (15 to 20 µl). The level of radioactivity of 1 µl of the labelled radioactive substrate in 500 µl water was counted using a Bioscan QC-2000.

2.20.2 *In vitro* pre-mRNA splicing assays

Reaction mixes were made up of 1X splice buffer (2 mM ATP, 2.5 mM magnesium chloride, 300 mM potassium phosphate pH 7.0), 3% PEG6000, 4 µl BJ2168 yeast whole cell extract and 2.75 µl of varying ratios of N-terminal protein fragment and water. 0, 3.96, 6.6, 9.24, 11.88 and 14.52 µg Snu114 1-122 dialysed with buffer D made up to a volume of 2.75 µl with water where necessary, were added to splicing reactions. Reactions were set up containing dialysis buffer D and water in the volumes outlined above as a negative control. Reactions were incubated at 23°C for 25 minutes. 0.25 µl ^{32}P body labelled actin pre-mRNA (262,000 dpm/µl) was added to each reaction resulting in a reaction volume of 10 µl, before incubating at 23°C for a further 15 minutes. Splicing reactions were stopped with the addition of 0.5 volumes of stop mix (1 mg/ml proteinase K, 50 mM EDTA, 1% SDS), and 15 minutes incubation at 37°C. 200 µl of splicing diluent (300mM sodium acetate pH 5.3, 1mM EDTA 0.1% SDS, 25µg/ml tRNA) and 200 µl of phenol-chloroform-isoamyl alcohol (PCA) were

added to each reaction. Reactions were vortexed and spun at 15600 g for 2 minutes. The aqueous phase from each reaction was transferred to a new tube. RNA was precipitated with the addition of 2.5 volumes of 100% ethanol, and incubation at -20°C for at least 30 minutes. Reactions were then spun at 15600 g for 5 minutes. The supernatant was aspirated off and RNA pellets were redissolved in 1 µl water and 3.5 µl formamide loading buffer. Samples were heated at 90°C for 5 minutes and ran on an 8% acrylamide/6 M urea gel, for 2 hours 15 minutes at 32 W in 1X TBE (45 mM Tris-borate, 1 mM EDTA). Gels were fixed in 10% methanol, 10% acetic acid, and dried onto filter paper. The dried gel was exposed to autoradiography film (Fuji).

2.21 Investigating RNA binding properties of protein fragments

2.21.1 End labeled snRNAs

1 µl of 1 µM *in vitro* transcribed UpG-primed snRNA was labeled in 10 µl reactions containing 1X PNK buffer (NEB), 3000 Ci/mmole, γ^{32}P-ATP (Amersham), 0.5 µl RNasin, 5 units T4 PNK (NEB). Reactions were incubated at 37°C for 1 hour. 1 µl 0.5M EDTA, 80 µl water, 100 µl citrate buffered phenol were added to each reaction. Reactions were vortexed for 2 minutes, and centrifuged at 15600 g for 2 minutes. The aqueous phase was removed and to this 0.2 volumes of 5M ammonium acetate, 20 µg tRNA, and 2.5 volumes of 100% ethanol were added. RNA was precipitated at -20 °C overnight. RNA was pelleted by centrifugation at 15600 g for 5 minutes, washed in 95% ethanol and resuspended in water to give 5000 dpm/µl when counted in Bioscan QC 2000 monitor.

2.21.2 Electrophoretic Mobility Shift Assay (EMSA)

Either 0, 5 or 10 µM 6His-tagged Snu114 1-122 in 50 mM sodium phosphate, pH 6.2, 100 mM sodium chloride, was incubated in 10 µl reactions containing 1 mg/ml tRNA, 2 mM HEPES-KOH, 15 mM potassium chloride, 150 µM magnesium chloride, 20 µM EDTA, 0.01% Triton X-100, and 0.5 µl ^{32}P end labeled RNA at 5000 dpm/µl. Reactions were incubated at room temperature for 10 minutes. 0.2 volumes of glycerol loading dye was added to each reaction. Complexes were resolved on a native 4% acrylamide, 6% glycerol gel at 150 V for 4 hours 46 minutes at 4°C, in 1X TBE (45 mM Tris-borate, 1 mM EDTA).

2.22 Construction of Snu114 N-terminal mutants

Single stranded DNA was made pRS413-Snu114 and used in oligomutagenesis using primers outlined in Table 2.3. Mutagenesis reactions were transformed into competent, XL1Blue MRF' cells. Plasmid DNA was isolated from the resulting colonies and sequenced to identify plasmids containing the correct mutation.

2.23 Testing viability and temperature sensitivity of mutants

Colonies were picked from transformation plates and grown overnight in appropriate selective medium at 30°C. Cells were diluted to OD_{600}=1 in water. This culture was subjected to four serial, 1 in 5 dilutions in water. 5 µl of each dilution was spotted onto the appropriate 5FOA plates and incubated at RT or 30°C for 3 days or 16°C or 37°C for 4 days.

2.24 Construction of U5 snRNA mutants in U5 + ins

Single stranded DNA was made pROK4 (U5 + ins) and used in oligomutagenesis using primers outlined in Table 2.3. Mutagenesis reactions were transformed into competent,

XL1Blue MRF' cells. Plasmid DNA was isolated from the resulting colonies and sequenced to identify plasmids containing the correct mutation.

2.24.1 Dideoxy DNA sequencing

When next day sequencing results were required sequencing was carried out using the Sequenase™Version 2.0 DNA Sequencing kit from USB. 5 μg plasmid DNA was denatured at 37°C in 0.2 M sodium hydroxide, 0.2 M EDTA for 30 minutes. 0.1 volumes of 3 M sodium acetate, pH 5.3 and 2.5 volumes of 100% ethanol were added and DNA was precipitated at -20°C for 30 minutes. DNA was pelleted by centrifugation at 15600 g for 5 minutes, washed with 95% ethanol and repelleted. DNA was resuspended in 7 μl water and 0.5 pmol U5 RT primer. The primer was annealed in 1X Sequanase reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM magnesium chloride, 250 mM sodium chloride) by heating to 65°C for 2 minutes and cooling to 30°C. For extension 1 μl 0.1M DTT, 2 μl labeling mix (1.5 μM of each dGTP, dCTP, dTTP), 5 μCi α35S-dATP (Amersham, 1000 Ci/mmol) and 3.23 untis T7 Sequenase DNA polymerase were added and reactions were incubated at room temperature for 5 minutes. This was terminated by adding 3.5 μl of sequencing reaction to 2.5 μl termination mix (8 μM of either ddGTP, ddCTP, ddTTP or ddATP, 80 μM of each dTNP and 50 mM sodium chloride) and incubated at 37°C for 5 minutes. Reactions were stopped with the addition of 4 μl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and incubation on ice. Reactions were heated to 90°C for 5 minutes and loaded onto a 7% acrylamide gel and ran at 32 W for 2 hours. Gels were fixed in 10% acetic acid and 10% methanol and dried. Dried gels were exposed on autoradiography film (Fuji) overnight. Products of mutagenesis were confirmed by sequencing.

2.25 Immunoprecipitation of TAP-tagged proteins and associated RNA from yeast extracts

50 µl of rabbit IgG agarose beads (Sigma) were washed 3 times in IPP150. The final wash was removed and 100 µl yeast whole cell extract containing TAP-tagged protein was added along with 300 µl of IPP150 (10 mM Tris-Cl pH 8, 150 mM sodium chloride, 0.1% IGEPAL), then incubated at 4°C for 2.5 hours on a roller. Beads were washed 4 times with 1 ml IPP150, the last wash was removed and 400 µl Splicing Diluent and 400 µl PCA were added. Samples were vortexed for 2 minutes and spun for 2 minutes. The aqueous phase was removed and added to an equal volume of PCA. This was repeated 2 more times. The final supernatant was transferred to a new tube, 2 µg tRNA and 2.5 volumes of 100 % ethanol were added. RNA was precipitated at -20°C for 30 minutes. RNA was spun down, then washed in 96 % ethanol and resuspended in water for use in primer extension.

2.26 Immunoprecipitation of Prp8p and associated RNA from yeast extracts using Prp8p antibodies

40 mg Protein A Sepharose^{TM} CL-4B beads (GE Healthcare) was washed 4 times in water, and resuspended in 600 µl IPP150 without IGEPAL (10 mM Tris-Cl pH 8, 150 mM sodium chloride). 70 µl of this was used per reaction. 8 µl Prp8p antibody was added to each reaction and incubated at 23°C for 2 hours. Beads were washed three times with IPP150 without IGEPAL. The final wash was removed and 150 µl yeast extract and 150 µl IPP150 without IGEPAL were added. Reactions were incubated on a roller at 4°C for two hours. Beads were washed four times with IPP150 without IGEPAL. The last wash was removed and 400 µl Splicing Diluent and 400 µl PCA were added. Samples were vortexed for 2 minutes and centrifuged at 15600 g for 2 minutes. The aqueous phase was removed and added to an equal

volume of PCA, and vortexed and centrifuged again. This was repeated 2 more times. The final supernatant was transferred to a new tube with 2 µl tRNA (10 mg/ml) and 2.5 volumes of 100% ethanol. RNA was precipitated at -20°C for 30 minutes. RNA was spun down, then washed in 96 % ethanol and resuspended in water for use in primer extension.

2.27 Preparation of RNA from whole cell yeast extracts

25 µl yeast extract was added to 125 µl water and 50 µl proteinase K stop mix. Reactions were incubated at 37°C for 15 minutes. 200 µl PCA was added and reactions were vortexed and spun at 15600 g for 2 minutes. Aqueous phase was removed and added to an equal volume of PCA, vortexed and spun. This was repeated two more times. Aqueous phase was added to 2.5 volumes of 100% ethanol and RNA was precipitated at -20°C for at least 30 minutes. RNA was pelleted by centrifugation at 15600 g for 5 minutes. RNA was washed with 95% ethanol and resuspended in 20 µl water.

2.28 Primer extension analysis

2.28.1 5' End Labelling of Oligonucleotide Primer

10 µl labelling reactions were set up containing 1 µl of 1 mM primer, 2 µl gamma ^{32}P-ATP (3000 Ci/mmole) (Heatmann Analytic), 1X PNK buffer (NEB), 10 units T4 PNK (NEB) and dH_2O. Reactions were incubated at 37°C for 30 minutes. 0.1 volume of 0.5 M EDTA, 10 volumes of TE buffer and TE Phenol were added and reactions were vortexed for 2 minutes and spun for 2 minutes at 15600 g. The aqueous phase was transferred to a new tube and 0.2 volumes of 5 M ammonium acetate, 20 µg tRNA, and 2.5 volumes of 100 % ethanol, then precipitated at -20°C for at least 1 hour. Reactions were spun for 5 minutes at 15600 g, pellets were washed in 96 % ethanol and resuspended in 20 µl water.

2.28.2 Primer Extension

All RNA from TAP tag or antibody purification reactions were used in a single primer extension reaction. When carrying out primer extension on RNA purified from whole cell yeast extracts 0.5 µl of RNA was used in each primer extension. 10 µl reactions were set up containing RNA, radiolabelled primer (0.5 µl of U2 RT all, U4 RT all and U6 RT all and 1 µl U1 136 RT all and 1.5 µl U5 RT) and 1x RT buffer (Roche). Reactions were heated to 90°C and cooled to 41°C. Reactions were increased to 20 µl with the addition of 1x RT buffer (Roche), 7.35 µl dNTP/DTT mix (1 mM each dNTP, 10 mM DTT), 10 units RNAsin (Promega), 3.3 units AMV RT (Roche). Reactions were incubated at 41°C for 30 minutes. 180 µl Splicing Diluent and 200 µl were added, reactions were vortexed and spun at 15600 g for 2 minutes. The aqueous phase was transferred to a new tube and precipitated with the addition of 2.5 volumes of 100% ethanol and incubation at -20°C for 1 hour. Primer extension products were spun down and resuspended in 1 µl water and 4 µl formamide loading dye and run out on a long 6% sequagel at 32 W for 2 hours. Gels were fixed in 10% actetic acid, 10% methanol for 10 minutes and dried. Dried gels were either exposed to autoradiography film (Fuji) or exposed to phosphorimaging screen (Fuji, BAS cassette 2040) for quantification.

2.28.3 Quantification of primer extension analysis

Primer extensions investigating the effects of U5 mutations on protein association were quantitated by phosphorimaging. Dried gels were exposed to a phosphorimaging screen (Fuji, BAS cassette 2040) overnight and scanned on a BioRad Molecular Imager FX. The wild type U5 snRNA band was used a loading control. Background readings were subtracted from all values. All data collected for U5 + ins mutants were normalized, where U5 + ins was equal to 1 and the reading for the lane containing only wild type U5 (not U5 + ins) was equal to 0.

Experiments were repeated in triplicate, expect in case of the experiment investigating the effects of deletions in the 3' side of U5 snRNA IL1 on associations of Snu114p, which has only been repeated twice.

2.29 Construction of *BRR2* mutants

Single stranded DNA was made pRS413-Brr2 and used in oligomutagenesis using primers outlined in Table 2.3. Mutagenesis reactions were transformed into competent, XL1Blue MRF' cells. Plasmid DNA was isolated from the resulting colonies and sequenced to identify plasmids containing the correct mutation.

Table 2.1 Bacterial strains

Strain	Genotype	Use
XL1-Blue MRF'	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 end A1 supE44 thi-1 recA1 gyrA96 relA1 lac [F'proAB $lacI^{q}ZΔM15$ Tn10 (tet^{r})]	Transformation of ligations reactions for blue white colour selection and isolation of plasmids for sequencing
BL21 (DE3) (Novagen)	F– ompT hsdSB(rB–, mB–) gal dcm (DE3)	Expression of recombinant Snu114p fragments
Rosetta 2 (DE3) (Novagen)	F- ompT hsdSB(rB- mB-) gal dcm (DE3) pRARE2 (CamR)	Expression of recombinant Snu114p fragments
BW313	Hfr lysA- dut^{-} ung^{-} thi^{-1} recA spoT1	Production of single stranded DNA

Table 2.2 Yeast strains

Strain	Genotype	Use	Source
SNU114/U5 KO	MATa; his3Δ200; leu2Δ0; trp1Δ63; ura3Δ0; YKL173w::kanMX4; snr7::kanMX4; pRS416-Snu114-U5	Synthetic lethal screen of *SNU114* and U5 snRNA mutants	Provided by RT O'Keefe
U5 KO	MATa; ura3-52; trp1Δ63; leu2Δ1; his3Δ200;GAL2; snr7::kanMX6; pRS416-U5	Testing the viability of U5 snRNA mutants *in vivo*	Provided by RT O'Keefe
BJ2168	MATα; prc1-407; prb1-1122; pep4-3; leu2; trp1; ura3-52; GAL2	In vitro splicing, immunoprecipitation of Prp8p and transformation with U5 snRNA mutants for analysis of Prp8p association with U5 snRNA mutants	LGC Promochem
Brr2-TAP	BJ2168 containing chromosomal TAP-tagged *BRR2*	Immunoprecipitation of Brr2p and transformation with U5 snRNA mutants for analysis of Brr2p association with U5 snRNA mutants	Provided by RT O'Keefe
Snu114-TAP	BJ2168 containing chromosomal TAP-tagged *SNU114*	Immunoprecipitation of Snu114p and transformation with U5 snRNA mutants for analysis of Snu114p association with U5 snRNA mutants	Provided by RT O'Keefe
Prp21-TAP	BJ2168 containing chromosomal TAP-tagged *PRP21*	Immunoprecipitation of Prp21p	Provided by RT O'Keefe
BRR2/U5 KO	MATa; ura3-52; his3Δ200; leu2Δ0; YER172C::kanMX4; snr7::hphNT1; pRS416-Snu114-U5	Synthetic lethal screen of *BRR2* and U5 snRNA mutants	Provided by RT O'Keefe

Table 2.3 Oligonucleotide primers

Name	Use	Sequence in 5' to 3' direction
Snu114 N-term forward	Amplification of all *SNU114* 1-122 and 1-400 fragments	GGAATTCCATATGGAAGGTGACGATTTATTCG
Snu114 1-122 reverse (pET28a)	Amplification of *SNU114* 1-122 fragment for insertion into pET28a expression vector	GGCGGCCGTTAAATAGTCTCGATTATATCTAGTCTTCG
Snu114 1-400 reverse (pET24b)	Amplification of *SNU114* 1-400 fragment for insertion into pET24b expression vector	GGCGGCCGCTAACAAATTCTTTAATTTATCTTTCTCC
Snu114 N 1-400 reverse (pET28a)	Amplification of *SNU114* 1-400 fragment for insertion into pET28a expression vector	GGCGGCCGTTATAACAAATTCTTTAATTTATCTTTCTCC
Snu114 833-1008 forward	Amplification of all *SNU114* 833-1008 fragment	GGAATTCCATATGAAAAGTCAAATTATTCCGC
C-term reverse (pET24b)	Amplification of *SNU114* 833-1008 fragment for insertion into pET24b expression vector	GGCGGCCGCCGGTACTAAGCCATTTTCTC
C-term reverse (pET28a)	Amplification of *SNU114* 833-1008 fragment for insertion into pET28a expression vector	GGCGGCCGTCACGGTACTAAGCCATTTTCTC
Snu114 N1	Mutagenesis of pRS413-Snu114 to produce Snu114 N1 mutant	ATGGAAGGTGACGCTTTATTCGCTGCTTTTGGAGCTTTGATCGGAGTTG
Snu114 N2	Mutagenesis of pRS413-Snu114 to produce Snu114 N2 mutant	GGAGTTGATCCTTTTGCTGCTGCTGCTGCTGCTGCTGTGCTGGATGAGC
Snu114 N3	Mutagenesis of pRS413-Snu114 to produce Snu114 N3 mutant	GAAGAAAGTGTGCTGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTTTTGAGGGGAGCGGC
Snu114 N4	Mutagenesis of pRS413-Snu114 to produce Snu114 N4 mutant	TGGAAGTATTAATGGCTGCTGCTGCTGCTGCTGCTCCACAGACTCCACTG
Snu114 N5	Mutagenesis of pRS413-Snu114 to produce Snu114 N5 mutant	CAGTCACCACAGACTGCTCTGGTAGCTGCTGTTGCTGCTGCTGCTGCTTTGCAAGAGCATAC
Snu114 N6	Mutagenesis of pRS413-Snu114 to produce Snu114 N6 mutant	TTAAAAAAAAACATTCCGGCTGCTGCTGCTGCTGCTGCTATGTTATCAATGGC
U1 RT 136	Primer extension for the detection of the U1 snRNA	GACCAAGGAGTTTGCATCAATGAC
U2 RT all	Primer extension for the detection of the U2 snRNA	GCCAAAAAATGTGTATTGTAAC
U4 RT all	Primer extension for the detection of the U4 snRNA	GGTATTCCAAAAATTCCCTACATAGTC
U5 RT	Primer extension for the detection of the U5 snRNA	AAAAATATGGCAGG CCTACAGTAACGG

Name	Use	Sequence in 5’ to 3’ direction
U6 RT all	Primer extension for the detection of the U6 snRNA	TCATCCTTATGCAGGG
U5 Δ75-83	Mutagenesis of ROK4 to produce U5 Δ75-83 mutant	GGCAAGAACCATAAGTTCTATAGGC
U5 Δ78-81	Mutagenesis of ROK4 to produce U5 Δ78-81 mutant	GCAAGAACCATGTTATAAGTTCTATAGG
U5 Δ79-80	Mutagenesis of ROK4 to produce U5 Δ79-80 mutant	CAAGAACCATGTTTTATAAGTTCTATAG
U5 75-83 sub	Mutagenesis of ROK4 to produce U5 75-83 sub mutant	GGCAAGAACCATCAAGCAATAAAGTTCTATAGG
U5 Δ92-102	Mutagenesis of ROK4 to produce U5 Δ92-102 mutant	CCGGATGGTTCTAGAACCATGTTCG
U5 Δ92-95	Mutagenesis of ROK4 to produce U5 Δ92-95 mutant	GGTTCTGGTAAAAAGAACCATGTTGC
U5 Δ96-99	Mutagenesis of ROK4 to produce U5 Δ96-99 mutant	GATGGTTCTGGTGGCAAGAACCAT
U5 Δ99-120	Mutagenesis of ROK4 to produce U5 Δ99-102 mutant	CCGGATGGTTCTAAAGGCAAGAACC
U5 Δ111-113	Mutagenesis of ROK4 to produce U5 Δ111-113 mutant	GCATACTTCTACAACACCATGGTTCTGGTAAAAGG
U5 Δ111-112	Mutagenesis of ROK4 to produce U5 Δ111-112 mutant	GCATACTTCTACAACACCCATGGTTCTGGTAAAAGG
U5 Δ111	Mutagenesis of ROK4 to produce U5 Δ111 mutant	GCATACTTCTACAACACCCGATGGTTCTGGTAAAAGG
Brr2-R295I	Mutagenesis of pRS413-Brr2 to produce Brr2-R295I mutant	TGACCGAAGTTTAATCTGCAAAAAAAATTC
Brr2-E610G	Mutagenesis of pRS413-Brr2 to produce Brr2-E610G mutant	TATGTCCCATTTCCCTGGCGTAGACAC
Brr2-P841L	Mutagenesis of pRS413-Brr2 to produce Brr2-P841L mutant	CACTGTATGAGCCAATAGATTAACACC
Brr2-G873L	Mutagenesis of pRS413-Brr2 to produce Brr2-G873L mutant	TCTGCCTGCTCTCAATAACATTTGAAG
Brr2-E610G	Mutagenesis of pRS413-Brr2 to produce Brr2-E610G mutant	TATGTCCCATTTCCCTGGCGTAGACAC
Brr2-E909K	Mutagenesis of pRS413-Brr2 to produce Brr2-E909K mutant	AACAAATTGCGATTTTATTGGTAATTG
Brr2-N1104L	Mutagenesis of pRS413-Brr2 to produce Brr2-N1104L mutant	CAATAACCTACCAGCCAATTGGTGAATAAACAC
Brr2-R1107A	Mutagenesis of pRS413-Brr2 to produce Brr2-R1107A mutant	AGCACGCAATAACGCACCAGCATTTTG
Brr2-R1107L	Mutagenesis of pRS413-Brr2 to produce Brr2-R1107L mutant	CATAGCACGCAATAACAAACCAGCATTTTGGTG
Brr2-F1149I	Mutagenesis of pRS413-Brr2 to produce Brr2-F1149I mutant	GTCCTTTAAGACAGATTAAAACATGCCCTGTC

Name	Use	Sequence in 5’ to 3’ direction
Brr2-G1375D,K1376N	Mutagenesis of pRS413-Brr2 to produce Brr2-G1375D,K1376N mutant	AGCCATAGCTGTATTATCTGTGCCCTTTCC
Brr2-D1474G	Mutagenesis of pRS413-Brr2 to produce Brr2-D1474G mutant	CTCATGAGCGTCACCATATATCATTAA

Table 2.4 Plasmids

Plasmid	Description/Use	Source
pRS413-Snu114	Yeast shuttle vector pRS413 (HIS3) containing SNU114 Template for PCR amplification of SNU114 fragments Construction of Snu114 N-terminal mutants Positive control in synthetic lethal screen of SNU114 and U5 snRNA mutants	(Frazer et al., 2009)
pBluescript II KS+	Blue-white screening and isolation of plasmids for sequencing of Snu114 fragments	Stratagene
pET24b	T7 promoter based expression vector Expression of Snu114p fragments	Novagen
pET28a	T7 promoter based expression vector Expression of Snu114p fragments	Novagen
pET28a-Snu114 833-1008	pET28a containing SNU114 fragment for expression amino acids 833-1008 with an N-terminal 6His tag	This study
pET24b-Snu114 1-400	pET24b containing SNU114 fragment for expression amino acids 1-400 with a C-terminal 6His tag	This study
pET28a-Snu114 1-400	pET28a containing SNU114 fragment for expression amino acids 1-400 with an N-terminal 6His tag	This study
pET28a-Snu114 1-122	pET28a containing SNU114 fragment for expression amino acids 1-122 with an N-terminal 6His tag	This study
M571	Yeast shuttle vector pRS314 (TRP1) containing the SNR7 gene for U5 snRNA Positive control in U5 snRNA synthetic lethal tests	(O'Keefe et al., 1996; Sikorski and Hieter, 1989)
Snu114 N1	pRS413-Snu114 containing mutations resulting in the substitution of amino acids 5 to 12 for alanine residues	This study
Snu114 N2	pRS413-Snu114 containing mutations resulting in the substitution of amino acids 20 to 26 for alanine residues	This study
Snu114 N3	pRS413-Snu114 containing mutations resulting in the substitution of amino acids 29 to 38 for alanine residues	This study
Snu114 N4	pRS413-Snu114 containing mutations resulting in the substitution of amino acids 79 to 85 for alanine residues	This study
Snu114 N5	pRS413-Snu114 containing mutations resulting in the substitution of amino acids 89, 92, 93 and 95 to 99 for alanine residues	This study
Snu114 N6	pRS413-Snu114 containing mutations resulting in the substitution of amino acids 115 to 122 for alanine residues	This study

Plasmid	Description/Use	Source
pRS413	Yeast HIS3 marked shuttle vector Negative control in synthetic lethal screen of SNU114 and BRR2 mutants	(Sikorski and Hieter, 1989)
pRS414	Yeast TRP1 marked shuttle vector Negative control in synthetic lethal screen of U5 snRNA mutants	(Sikorski and Hieter, 1989)
U5 ΔC94,C95	M571 containing ΔC94,C95 mutant SNR7 gene	(Frazer et al., 2009)
U5 ΔU96,U97	M571 containing ΔU96,U97 mutant SNR7 gene	(Frazer et al., 2009)
U5 ΔC112,G113	M571 containing ΔC112,G113 mutant SNR7 gene	(Frazer et al., 2009)
ROK4/U5 + ins	M571 containing a 20 nucleotide insert in stem 2	Provided by RT O'Keefe
U5 + ins-Δ75-83	U5 + ins plasmid containing Δ75-83 mutant SNR7 gene	This study
U5 + ins-Δ78-81	U5 + ins plasmid containing Δ78-81 mutant SNR7 gene	This study
U5 + ins-Δ79-80	U5 + ins plasmid containing Δ79-80 mutant SNR7 gene	This study
U5 + ins-Δ75-83	U5 + ins plasmid containing Δ75-83 mutant SNR7 gene	This study
U5 + ins-75-83 sub	U5 + ins plasmid containing mutant SNR7 gene with a sequence substitution of nucleotides 75 to 83	This study
U5 + ins-Δ92-102	U5 + ins plasmid containing Δ92-102 mutant SNR7 gene	This study
U5 + ins-Δ92-95	U5 + ins plasmid containing Δ92-95 mutant SNR7 gene	This study
U5 + ins-Δ96-99	U5 + ins plasmid containing Δ96-99 mutant SNR7 gene	This study
U5 + ins-Δ99-102	U5 + ins plasmid containing Δ99-102 mutant SNR7 gene	This study
U5 + ins-Δ111-113	U5 + ins plasmid containing Δ111-113 mutant SNR7 gene	This study
U5 + ins-Δ111-112	U5 + ins plasmid containing Δ111-112 mutant SNR7 gene	This study
U5 + ins-Δ111	U5 + ins plasmid containing Δ111 mutant SNR7 gene	This study
pRS413-Brr2	Yeast shuttle vector pRS413 (HIS3) containing BRR2. Construction of BRR2 mutants. Positive control in synthetic lethal screen of BRR2 and U5 snRNA mutants	Provided by RT O'Keefe
pRS415-U5	Yeast shuttle vector pRS415 (LEU2) containing the SNR7 gene for U5 snRNA. Positive control in synthetic lethal screen of BRR2 and U5 snRNA mutants	Provided by RT O'Keefe

Plasmid	Description/Use	Source
pRS415-U5 ΔC79-A81	pRS415-U5 containing ΔC79-A81 mutant SNR7 gene	Provided by RT O'Keefe
pRS415-U5 ΔG93	pRS415-U5 containing ΔG93 mutant SNR7 gene	Provided by RT O'Keefe
pRS415-U5 ΔC94,C95	pRS415-U5 containing ΔC94,C95 mutant SNR7 gene	Provided by RT O'Keefe
pRS415-U5 ΔU96,U97	pRS415-U5 containing ΔU96,U97 mutant SNR7 gene	Provided by RT O'Keefe
pRS415-U5 A100U,C101G	pRS415-U5 containing A100U,C101G mutant SNR7 gene	Provided by RT O'Keefe
pRS415-U5 ΔC111	pRS415-U5 containing ΔC111 mutant SNR7 gene	Provided by RT O'Keefe
pRS415-U5 ΔC112,G113	pRS415-U5 containing ΔC112,G113 mutant SNR7 gene	Provided by RT O'Keefe
pRS415	Yeast LEU2 marked shuttle vector Negative control in synthetic lethal screen of BRR2 and U5 snRNA mutants	(Sikorski and Hieter, 1989)
Brr2-R295I	pRS413-Brr2, containing mutant BRR2 with an arginine to isoleucine substitution at position 295	This study
Brr2- E610G	pRS413-Brr2, containing mutant BRR2 with a glutamic acid to glycine substitution at position 610	This study
Brr2- P841L	pRS413-Brr2, containing mutant BRR2 with a proline to leucine substitution at position 841	This study
Brr2- G873L	pRS413-Brr2, containing mutant BRR2 with a glycine to leucine substitution at position 873	This study
Brr2- E909K	pRS413-Brr2, containing mutant BRR2 with a glutamic acid to lysine substitution at position 909	This study
Brr2- N1104L	pRS413-Brr2, containing mutant BRR2 with an asparagine to leucine substitution at position 1104	This study
Brr2- R1107A	pRS413-Brr2, containing mutant BRR2 with an arginine to alanine substitution at position 1107	This study
Brr2-R1107L	pRS413-Brr2, containing mutant BRR2 with an arginine to leucine substitution at position 1107	This study
Brr2- F1149I	pRS413-Brr2, containing mutant BRR2 with a phenylalanine to isoleucine substitution at position 1149	This study
Brr2- G1375D,K1376N	pRS413-Brr2, containing mutant BRR2 with a glycine to aspartic acid and lysine to asparagine substitution at positions 1375 and 1376	This study
Brr2- D1474G	pRS413-Brr2, containing mutant BRR2 with an Aspartic acid to glycine substitution at position 1474	This study

Table 2.5 Antibodies

Antibody	Description
Rabbit anti-Snu114p (Eurogentec)	Used at 1:10,000 dilution and detected with goat anti-rabbit HRP conjugated (Pierce) secondary antibody at 1:5000 dilution
Monoclonal Mouse anti-poly His (Sigma)	Used at 1:10,000 dilution and detected with goat anti-mouse HRP conjugated (Pierce) secondary antibody at 1:1000 dilution
Polyclonal Rabbit anti-TAP (Open Biosystems)	Used at 1:1000 dilution and detected with goat anti-rabbit HRP conjugated (Pierce) secondary antibody at 1:5000 dilution
Anti-Prp8p-N terminal R1703 (provided by J Beggs)	Used at 1:5000 dilution and detected with protein A-HRP conjugated secondary antibody (Biorad) at 1:5000 dilution
Rabbit anti-G6PDH (Sigma)	Used at 1:20,000 dilution and detected with goat anti-rabbit HRP conjugated (Pierce) secondary antibody at 1:10,000 dilution

3 Structural and functional analysis of Snu114p

Snu114p is an essential *S.cerevisiae* protein involved in several aspects of pre-mRNA splicing, in particular U5-snRNP formation, U4/U6 unwinding and disassembly of the post splicing complex (Bartels et al., 2002; Bartels et al., 2003; Brenner and Guthrie, 2005, 2006; Fabrizio et al., 1997; Small et al., 2006). The exact roles of Snu114p in these processes have yet to be defined. It is currently thought that the GTP state of Snu114p affects the function of Brr2p in the control of U4/U6 unwinding and disassembly of the post splicing complex via U2/U6 unwinding, but the mechanisms behind the GTP binding and hydrolysis of Snu114p and how this is communicated to Brr2p are still elusive (Small et al., 2006). It has been proposed that Brr2p translocates along the U6 snRNA disrupting both the U4/U6 and U2/U6 base pairing, and that the G domain of Snu114p senses RNA/RNA interactions and transmits a signal activating the unwinding activities of Brr2p via protein-protein interactions (Achsel et al., 1998; Frazer et al., 2009; Liu et al., 2006; Small et al., 2006). It is clear that Snu114p is a key spliceosome component; however, it is also clear that there are still many unanswered questions surrounding the roles of Snu114p in pre-mRNA splicing.

To allow investigations into the functions of Snu114p it would be beneficial to obtain purified Snu114p, allowing a wide range of *in vitro* experiments, including binding assays, splicing assays and structural studies to be carried out. The expression and purification of the complete 1008 amino acids of Snu114p has been attempted previously in the lab, however the attempt was unsuccessful. As we have been unable to obtain full length purified Snu114p, and as the expression and purification of smaller protein fragments is often easier, it was decided that fragments of Snu114p would be expressed to allow structural and functional analysis of specific domains of Snu114p. To enable production of recombinant fragments of Snu114p,

bacterial constructs were produced to express three specific regions of Snu114p in *E.coli* cells (Figure 3.1).

3.1 Results

3.1.1 Expression of recombinant Snu114p C-terminal region (Snu114 833-1008)

The first region chosen for expression was the C-terminal 176 amino acids of Snu114p (Snu114 833-1008) (Figure 3.1). The C-terminal region of Snu114p contains amino acids not present in the C-terminus of EF-2 (Figure 3.2), and it is expected that these residues have a function specific to splicing (Brenner and Guthrie, 2005; Fabrizio et al., 1997). Overexpression of the C-terminus of Snu114p has been shown to result in growth inhibition (Akada et al., 1997). This dominant negative effect suggests that this region may be involved in protein or RNA interactions. It is already known that the C-terminus of human Snu114p interacts with human Prp8p and genetic interactions between the C-terminus of Snu114p and the U5 snRNA have also been identified (Frazer et al., 2009; Liu et al., 2006). A role for the C-terminus of Snu114p in spliceosome activation has been demonstrated, with a C-terminal deletion causing a block in U4 release and the identification of genetic interactions between the C-terminus of Snu114p and Prp28p, Brr2p and Prp8p (Brenner and Guthrie, 2005, 2006).

Obtaining purified Snu114 833-1008 fragment would allow investigations into the protein and RNA interactions of this region by carrying out experiments such as immunoprecipitations and electromobility shift assays. It would also allow structural studies of Snu114 833-1008, providing an insight into how the differences in amino acids present in the Snu114p C-terminus and EF-2, alter the structure compared to the predicted structure based on EF-2 (Bartels et al., 2003).

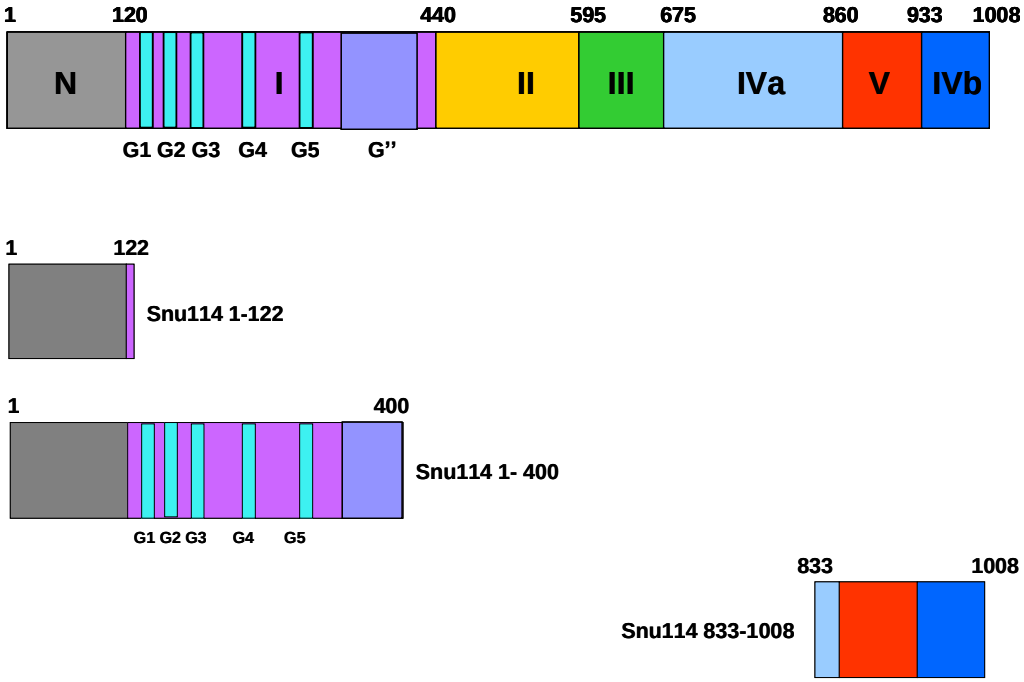


Figure 3.1 Domains of Snu114p and fragments expressed for structural and functional analysis. Snu114p is divided into domains I, II, III, IVa, V and IVb based upon sequence similarities with EF-2. Domain I, the G domain, contains the conserved elements G1 to G5 for GTP binding and hydrolysis. Snu114p also contains a unique N-terminal domain (amino acids 1-120) that is absent in EF-2. Three fragments of Snu114p (Snu114 1-122, 1-400 and 833-1008) were chosen for expression and purification to allow structural and functional analysis. Numbers above each bar represent amino acid numbers. The amino acids 121 and 122 were included in the N-terminla fragment because they are part of a conserved region of amino acids. The C-terminal 176 amino acids were chose fore xpersison because this includes the region of the C-terminus that causes a growth phenotype when over expressed (Akada et al., 1997). The N-terminal 400 amino acids were chosed for expression as this region contains the motifs G1 to G5 that are required for GTP binding and hydrolysis. Expression of the N-terminal 440 amino acids was attempted but was not successful (data not shown), so the expressed fragment was downsized to 400 amino acids, in an attempt to allow expression of this protein fragment.

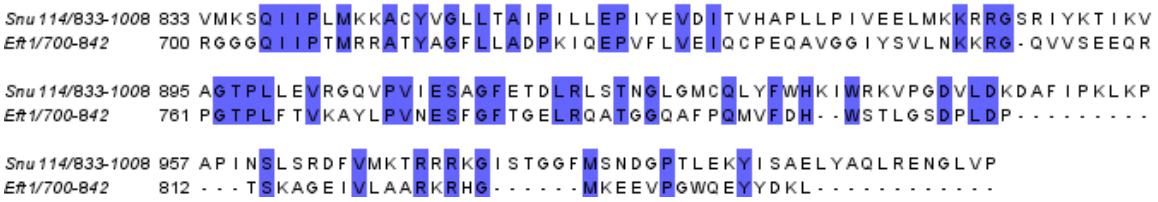


Figure 3.2 Alignment of the amino acid sequences of the C-termini of Snu114p and Elongation Factor 2 (EF2) in *S.cerevisiae*. The C-terminus of Snu114p contains several regions of amino acids that are absent in the homologue EF2 (encoded by Eft1 and Eft2 in yeast). The function of these amino acids in pre-mRNA splicing is currently unknown. Amino acids highlighted in blue are consereved between Snu114p and EF2. Alignment was produced using ClustalW and Jal view.

To allow studies to further define the role of the C-terminus of Snu114p in splicing, the Snu114 833-1008 fragment was expressed and purified from bacteria. Constructs expressing both N- and C-terminally 6His-tagged Snu114 833-1008 were produced. Test expressions of both constructs were carried out using the bacterial expression strains, BL21 (DE3) and Rosetta 2 (DE3). Rosetta 2 (DE3) cells contain a plasmid that expresses seven rare tRNAs, so expression of proteins requiring these tRNAs can be achieved. After carrying out test expressions and purifications from both strains grown at room temperature and 37°C, it was discovered that the best expression was of N-terminally 6His-tagged Snu114 833-1008 in BL21 (DE3) cells growing at room temperature (data not shown). The expression was scaled up to 1 litre and 6His-tagged Snu114 833-1008 fragment was purified. All purification steps were analyzed by electrophoresis (Figure 3.3). The expected size of N-terminally 6His-tagged Snu114 833-1008 is 21.84 kDa. A band of this size is present in fractions 4 to 6 (Figure 3.3, lanes 5 to 7), indicating that the purification was successful. However, Snu114 833-1008 expression was low and no highly expressed protein of 21.84 kDa was seen in the bacterial extract (lane 8). To obtain enough protein to allow further analysis of Snu114 833-1008, large scale culture of bacterial cells would be required, and this was outside of the capablities of our academic lab.

3.1.2 Expression of recombinant Snu114p N-terminal region and G domain (Snu114 1-400)

The second region chosen for expression and purification was the first 400 amino acids of Snu114p (Snu114 1-400) (Figure 3.1). This includes the N-terminus and domain I containing consensus sequences for GTP binding and hydrolysis (Fabrizio *et al*., 1997; Brenner and Guthrie, 2006). Snu114p is the only GTPase currently known to be involved in pre-mRNA

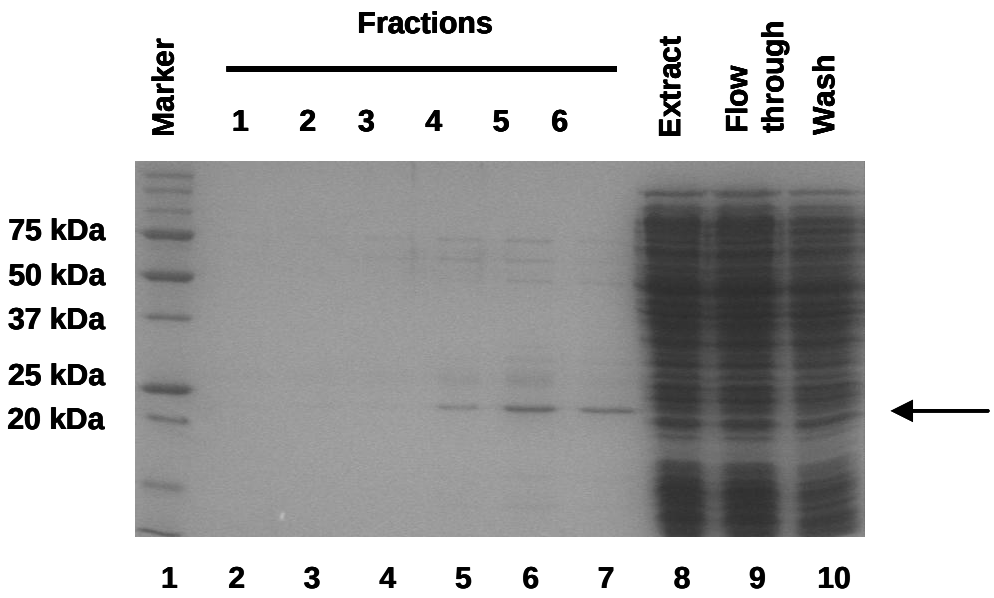


Figure 3.3 Purification of Snu114 833-1008. Coomassie blue stained 12% SDS-PAGE gel illustrating the steps in purification of N-terminally 6His-tagged Snu114 833-1008 from bacteria. Snu114 833-1008 was expressed in BL21 (DE3) cells grown in Overnight Express media at room temperature for 36 hours. Bacterial extract was produced (lane 8), and the extract was applied to a HIS-Select Nickel affinity column. Flow through (lane 9) and wash (lane 10) from the column purification were analysed. Purified protein was eluted with 250 mM imidazole and collected in 500 µl fractions (lanes 2 to 7). Lane 1 contains molecular weight marker. The arrow indicates the postion of the expected size of 6His-tagged Snu114 833-1008.

splicing; It is well documented that GTP binding and hydrolysis are important for the functions of Snu114p, with several mutation in the G domain of Snu114p resulting in lethal phenotypes (Bartels et al., 2003; Brenner and Guthrie, 2006; Fabrizio et al., 1997). It is known that the GTP state of Snu114p affects the activities of Brr2p in U4/U6 and U2/U6 unwinding in spliceosomal activation and disassembly, but it is not known how, or if, there is a common pathway controlling both unwinding events mediated by Snu114p (Small et al., 2006). The G domain of Snu114p has recently been implicated in sensing RNA/RNA interactions in the spliceosome, with genetic interactions between the G domain and U2, U4, U5 and U6 snRNAs being identified (Frazer et al., 2009). However, whether these interactions are direct or indirect, is still to be determined. It is also unknown if the GTP state of Snu114p effects any of its protein or RNA interactions. A further question surrounding the GTPase activity of Snu114p, is how the GTPase activity is controlled or activated.

Purification of Snu114 1-400 would allow investigation into, and further definition of, the protein and RNA associations of Snu114 1-400. Obtaining purified Snu114 1-400 would also allow experiments to determine if interactions with snRNAs are direct or indirect and investigation into how the GTP state of Snu114p is controlled. Structural studies of this domain may provide information on any conformational changes that occur within Snu114p due to GTP binding or hydrolysis.

To allow such investigations, constructs for Snu114 1-400 were produced to give N- and C-terminally 6His-tagged fragments. Small scale test expressions and purifications were carried out using BL21 (DE3) and Rosetta 2 (DE3) cells grown at room temperature and 37°C (Figure 3.4 and data not shown). Cell extract and eluted fractions were analysed by electrophoresis. The expected size of Snu114 1-400 when N-terminally 6His-tagged is 47.75 kDa and 46.86 kDa when C-terminally 6His-tagged. No proteins of these sizes are observed in

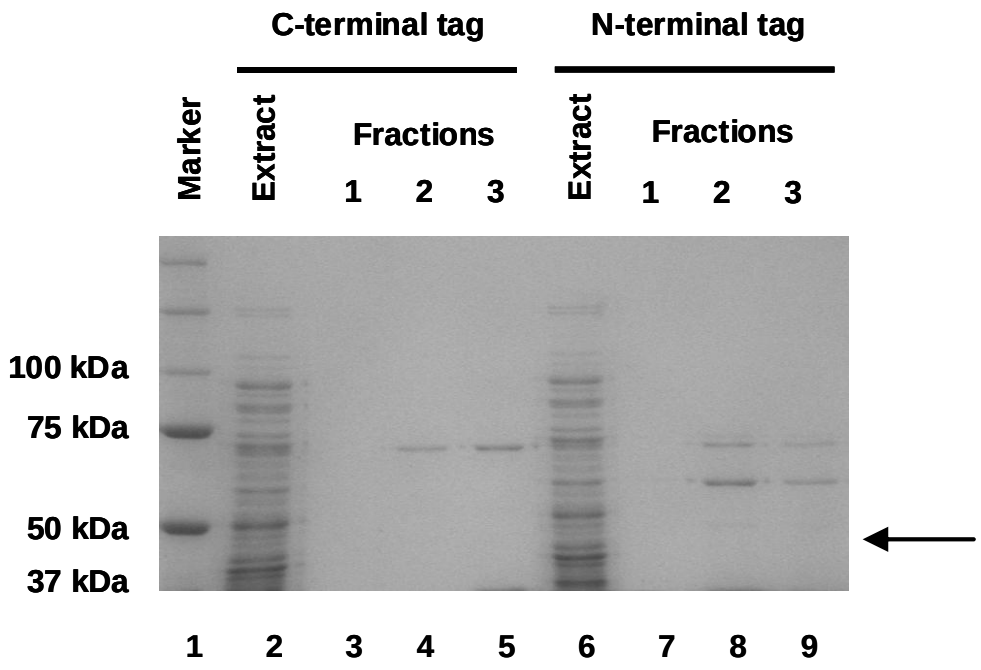


Figure 3.4 Purification of Snu114 1-400. Coomassie blue stained 12% SDS-PAGE gel illustrating the attempted purification of both N- and C-terminally 6His-tagged Snu114 1-400 from bacteria. BL21 (DE3) cells expressing either N- or C-terminally tagged Snu114 1-400 were grown in Overnight Express media at room temperature for 36 hours. Bacterial extracts were produced (lanes 2 and 6) and applied to a HIS-Select Nickel affinity column. Purified protein was eluted with 250 mM imidazole and collected in 500 µl fractions (lanes 3 to 5 and 7 to 9). Lane 1 contains a molecular weight marker. The arrow indicates the postion of the expected size of 6His-tagged Snu114 1-400.

the purified fractions (Figure 3.4, lanes 3 to 5 and 7 to 9). No strong bands of expressed proteins of 47.75 kDa or 46.86 kDa can be seen in the bacterial extract from either test (Figure 3.4, lanes 2 and 6). Expression and purification of Snu114 1-400, therefore, was not achieved.

3.1.3 Expression of recombinant Snu114p N-terminus (Snu114 1-122)

The third and final fragment chosen for expression was the N-terminal 122 amino acids of Snu114p (Snu114 1-122) (Figure 3.1). The N-terminus was chosen for study as deletion of this region causes a defect in U4/U6 unwinding during spliceosomal activation, but the exact role of the N-terminus in U4/U6 unwinding is unknown (Bartels et al., 2002). It is unclear if the N-terminus of Snu114p has a role in protein-protein or protein-RNA interactions, although a genetic interaction between the N-terminus of Snu114p and U5 and U6 snRNAs have been identified (Frazer et al., 2009). It is also unknown if the N-terminus of Snu114p has any intramolecular interactions with any other domains of Snu114p. Recent data has revealed that the N-terminus of Snu114p can be phosphorylated, bringing to light many questions surrounding the cause, purpose and timing of this phosphorylation (Gruhler et al., 2005). This region is of particular interest from a structural point of view due to its absence in EF-2, which shares homology with Snu114p. The N-terminal region of Snu114p does not feature in the current predicted structure of Snu114p, as it was created based on the structure of EF-2 (Fabrizio et al., 1997; Frazer et al., 2009). Aside from having no predicted structure for Snu114 1-122, the amino acid sequence gives no clues to either its structure or function, so structural analysis of this region would be both novel and of great interest.

The N-terminal 366 nucleotides of *SNU114* were cloned into an expression vector to give N-terminally 6His-tagged protein. Test expressions in BL21 (DE3) cells grown at 37°C were carried out, and good expression of Snu114 1-122 was observed (data not shown). Large

scale expression and purification of Snu114 1-122 was performed growing BL21 (DE3) cells at 37°C for 36 hours. All purification steps were analysed by electrophoresis (Figure 3.5). The expected size of N-terminally 6His-tagged Snu114 1-122 is 16.19 kDa. High expression of a protein of around 20 kDa was present in the bacterial extract (Figure 3.5, lane 15). A protein of 20kDa was also apparent in the purified fractions (Figure 3.5, lanes 2 to 10 and 12 to 14). Fractions 3 to 7 were pooled and dialysed resulting in a protein concentration of 5.28 mg/ml.

The purified Snu114 1-122 did appear slightly larger than the expected size of N-terminally 6His-tagged Snu114 1-122 of 16.19 kDa, so western blotting using either an anti-Snu114p antibody specific to the N-terminus or an anti-6His antibody, was carried out to confirm that the purified protein was 6His-tagged Snu114 1-122 (Figure 3.6). Samples of the purified Snu114 1-122 before and after dialysis (Figure 3.6, lanes 1, 2, 4 and 5), and a sample of the bacterial extract from bacteria expressing Snu114 1-122 (Figure 3.6, lanes 3 and 6), were tested by western blotting. The strong band migrating along side the 20 kDa molecular marker detected in all samples with both the anti-Snu114p and anti-His antibodies confirms the purified fragment is the recombinant 6His-tagged Snu114 1-122. Further confirmation of the identity of purified Snu114 1-122 was gained via mass spectrometry analysis as a more accurate determination of the mass of the protein fragment (Figure 3.7). The mass spectrometry detected a major peak of protein at 16.06 kDa. N-terminally 6His-tagged Snu114 1-122 is expected to be 16.19 kDa. The smaller than expected mass suggests that a single amino acid may have been lost due to degradation. In bacterial cells N-terminal methionine can be formylated and cleaved off teh peptide chain (Varshney and Rajbhandary, 1992). This is another possible reason for the smaller than expected protein fragment. The mass spectrometry results in combination with the western blot data, confirms that the purified fragment is Snu114 1-122. As Snu114 1-122 was expressed and purified in high abundance,

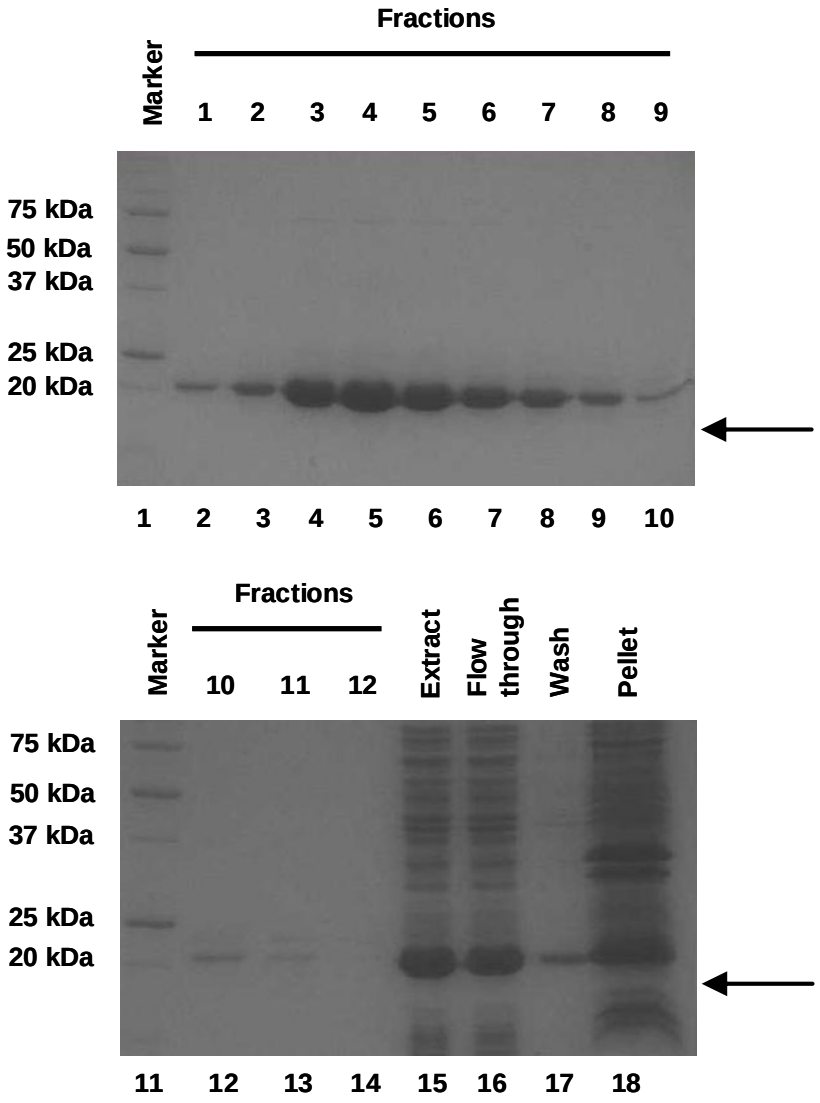


Figure 3.5 Purification of Snu114 1-122. Coomassie blue stained 12% SDS-PAGE gel illustrating the steps in purification of N-terminally 6His-tagged Snu114 1-122 from bacteria. Snu114 1-122 was expressed in BL21 (DE3) cells grown in PASM 5052 media at 37°C for 36 hours. Bacterial extract was produced (lane 15), and the extract was applied to a HIS-Select Nickel affinity column. Flow through and wash from the column purification were analysed (lanes 16 and 17). Purified protein was eluted with 250 mM imidazole and collected in 500 µl fractions (lanes 2 to 14). Insoluble protein from the bacterial extract was also analysed (lane 18). Lanes 1 and 11 contain molecular weight markers.

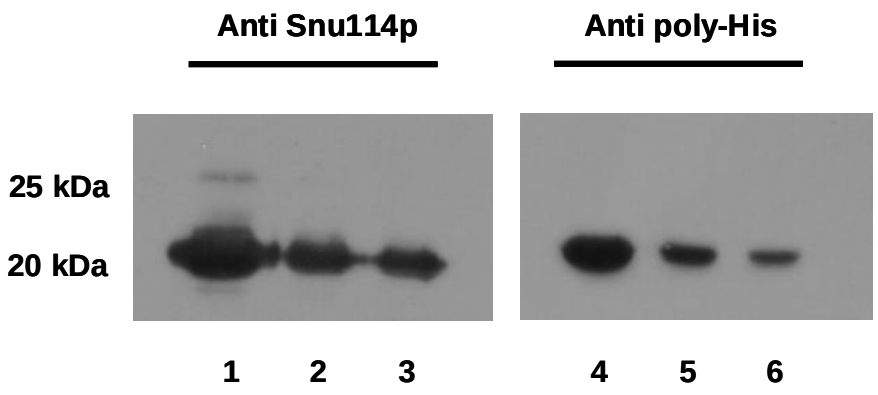


Figure 3.6 Western blotting to confirm the identity of purified 6His-tagged Snu114 1-122. Extract from bacteria expressing 6His-tagged Snu114 1-122 (lanes 3 and 6), and purified Snu114 1-122 before (lanes 1 and 4) and after dialysis (lanes 2 and 5) were applied to a 12% SDS-PAGE gel and transferred to nitrocellulose membrane. Western blotting was carried out with an anti-Snu114p antibody which binds to the proteins N-terminal 15 amino acids (lanes 1 to 3), and an anti-His antibody that binds the poly His-tag (lanes 4 to 6).

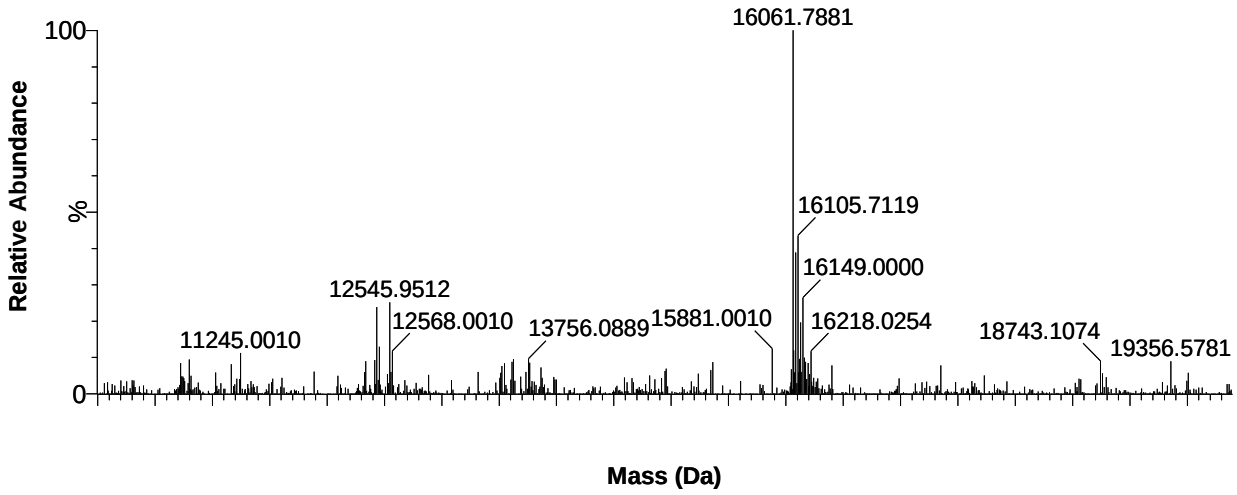


Figure 3.7 Mass spectrometry to confirm the mass of purified Snu114 1-122. For an accurate measurement of mass, mass spectrometry was carried out on a sample of purified then dialysed 6His-tagged Snu114 1-122 at 50 pmol/µl in 10mM Tris-HCl, pH 7.4, 150 mM NaCl. The X axis represents the mass of protein detected in Da (also displayed at the top of each peak). The Y axis is a measure of the presence of all products detected by mass spectrometry, with the most abundant protein being scored as 100 % and the amounts of all other products present being expressed as a percentage of that.

further structural and functional analysis of the protein fragment could be carried out.

3.1.4.1 Structural analysis of the N-terminal region of Snu114p

Solving the structure of a protein is an invaluable tool when trying to define its function, allowing identification of known structures or structural homology. The structure of Snu114p can be predicted based upon the structure of EF-2, due to sequence similarities. However, the N-terminal region of Snu114p is unique and is absent in EF-2 so cannot be included in the predicted structure (Fabrizio et al., 1997; Frazer et al., 2009; Jorgensen et al., 2002). As well as being absent in the close homologue of Snu114p, the sequence of the N-terminal region does not provide any clues to its structure or function. For this reason, attempts to determine the structure of the N-terminal region of Snu114p were made.

3.1.4.2 Crystallography to solve the structure of Snu114 1-122

Crystal growth trails were carried out to try and achieve crystal formation of the purified recombinant Snu114 1-122, to allow the structure of Snu114 1-122 to be solved. Crystal trials with Snu114 1-122 were set up using 384 different conditions for crystal growth. Of all the conditions tested only two resulted in crystal growth (E12 Classics suite- 0.1 M BICINE pH 9.0 and 2 M Magnesium chloride, and A7 SM1 suite- 0.2 M Magnesium chloride, 0.1 M Imidazole pH 8.0 and 35 % (v/v) MPD) (Figure 3.8, A and C). Crystals were removed from the trial plates and X-ray diffraction was attempted. X-ray diffraction was achieved (Figure 3.8, B and D), but the detection of strong sparse diffracted X-rays indicated that both crystals were salt, not protein.

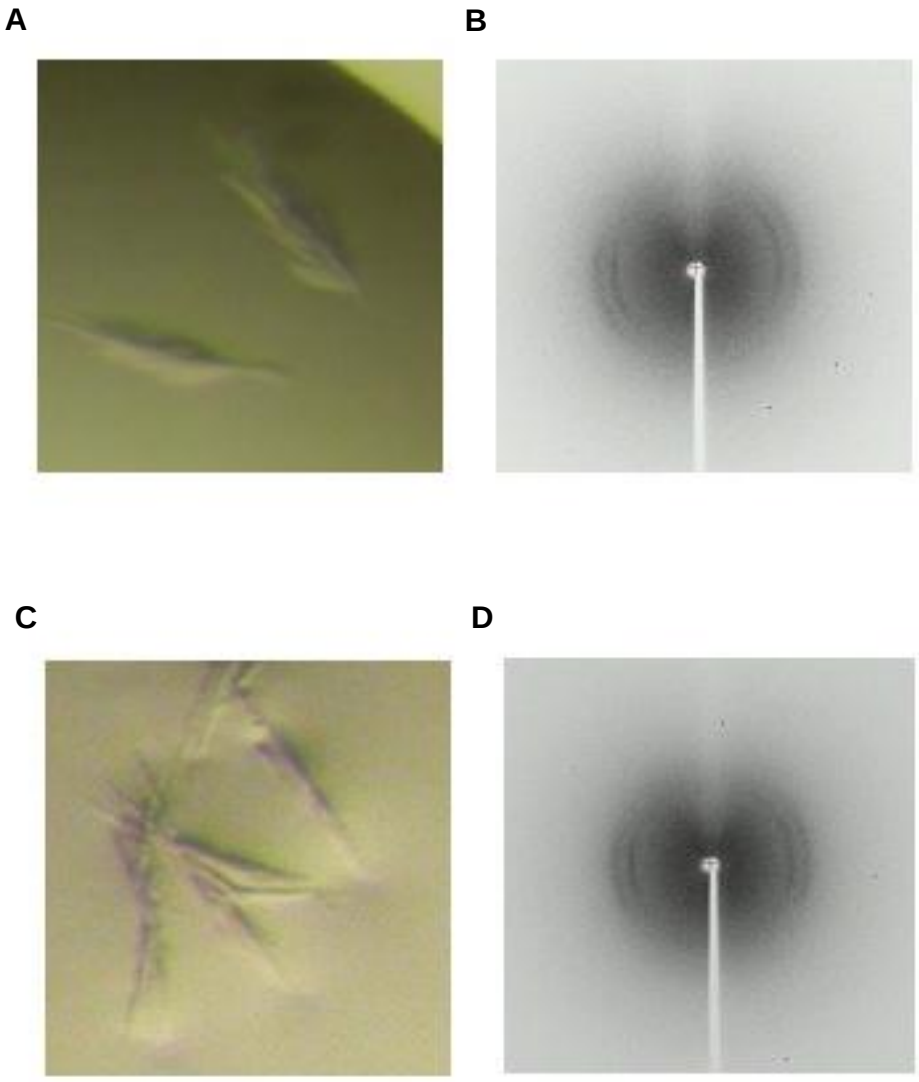


Figure 3.8 Candidate crystals from crystal growth trials of Snu114 1-122. Crystal growth trails were set up using 384 different conditions. Two conditions tested resulted in crystal growth. Panel A illustrates crystals formed using E12 Classic suite conditions (0.1 M BICINE pH 9.0 and 2 M Magnesium chloride). Crystals in panel A were subjected to X-ray diffraction. The collected diffraction pattern is displayed in panel B. Panel C illustrates crystals formed in A7 SM1 suite conditions (0.2 M Magnesium chloride, 0.1 M Imidazole pH 8.0 and 35 % (v/v) MPD). These crystals were also subjected to X-ray diffraction. The collected diffraction pattern is displayed in panel D.

3.1.4.3 Structural analysis of Snu114 1-122 by NMR

As the crystallisation trials did not yield protein crystals, the alternative approach of Nuclear Magnetic Resonance (NMR) spectroscopy was attempted. Preliminary experiments were carried out using unlabelled or isotopically normal Snu114 1-122 to determine if the protein fragment was stable and if any structure was detectable.

Unlabelled Snu114 1-122 was used in a 1D proton experiment in ^{1}H2O, which provides information on proton groups present in the protein, and also detects any labile, exchangeable backbone amide proton resonances. The experiment was carried out at 700 MHz at 2, 10, 15, 20 and 25°C, and the spectra remained almost unchanged at all temperatures indicating a thermodynamically stable protein (Figure 3.9). Amide NH protons appear to resonate with considerable overlap between 8.0 and 9.2 parts per million (ppm) in the spectra. Similarly, there is a lack of dispersed peaks in the aliphatic region, below 0.6 ppm, which indicates that there is little secondary structure in the protein fragment. 2D Nuclear Overhauser effect spectroscopy (NOESY) of the same sample was carried out to gain through space interactions within Snu114 1-122 (Figure 3.10). NOE is a result of spatial interactions between different nuclei, and this spectra reveals information on proximity of various nuclei by means of proton connectivities. This spectra revealed limited amide-amide proton connectivities, characteristic of α-helical type conformations.

Experiments were also carried using unlabeled Snu114 1-122 in 100 % $^{2}H_2O$. ^{2}H or deuterium H_2O was used as a solvent instead of $^{1}H_2O$, to remove exchangeable protons and observe only exchangeable C-H protons, simplifying the spectrum analysis. 1D proton (Figure 3.11) and 2D total correlation spectroscopy (TOCSY) (Figure 3.12) spectra with a mixing time of 75 ms were measured at 25°C. These experiments enabled detection of non-exchangeable proton resonances. The spectra did not show any slowly exchanging labile amide NH protons,

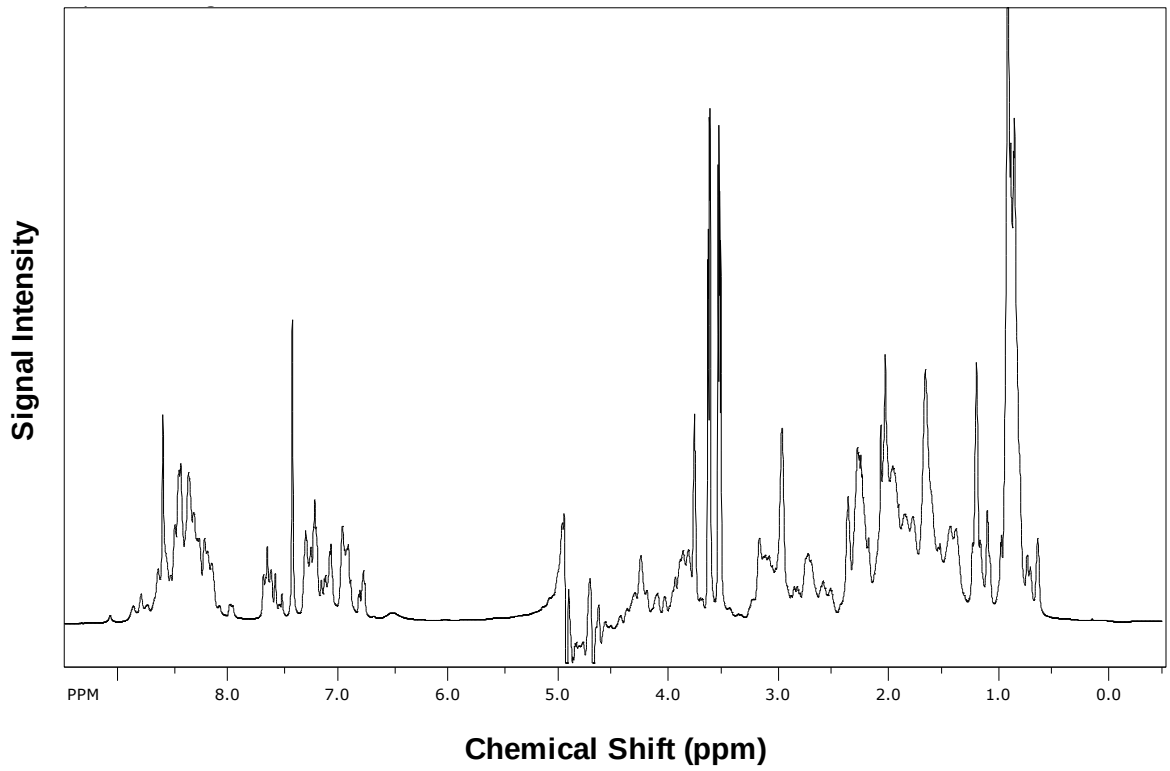


Figure 3.9 1H-NMR spectrum of unlabelled Snu114 1-122 at 10 °C. Figure illustrates 700 MHz 1H-NMR spectrum of unlabelled, isotopically normal Snu114 1-122 protein (0.7 mM) in 90% $1H_2O$+10% $2H_2O$, containing 50 mM sodium phosphate pH 6.2, 100 mM sodium chloride at 10°C. This experiment investigated the chemical groups present within Snu114 1-122 and was carried out at different temperatures to confirm that Snu114 1-122 is stable. Peaks corresponding to amide NH protons overlap beteween 8.0 and 9.2 ppm, and peaks in the aliphatic region below 6.0 ppm, indicating that there is little secondary structure in Snu114 1-122.

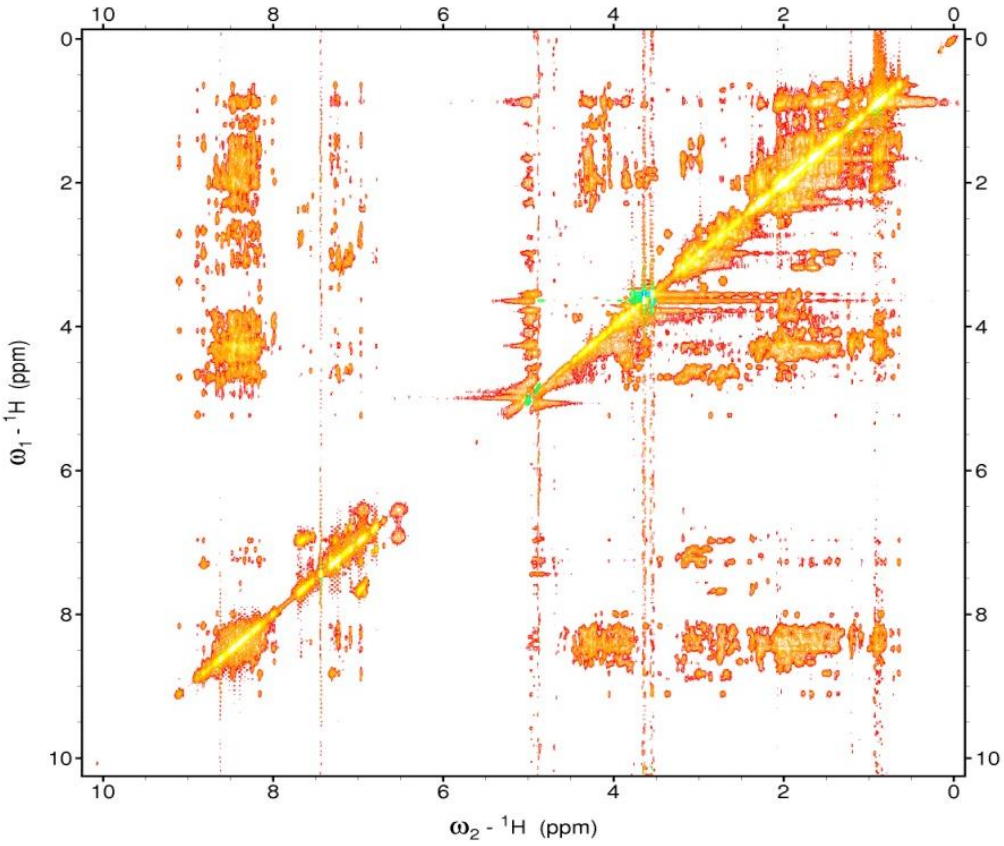


Figure 3.10 1H-NMR NOESY spectrum of unlabelled Snu114 1-122 at 10 °C. Figure illustrates 700 MHz 1H-NMR NOESY (mixing time=150 ms) spectrum of unlabelled, isotopically normal Snu114 1-122 (0.7 mM) in 90% $1H_2O$+10% $2H_2O$, containing 50 mM sodium phosphate pH 6.2, 100 mM sodium chloride at 10°C. This experiment investigates the spatial relationship between protons in different nuclei. This experiment revealed limited amide-amide proton connectivities within Snu114 1-122 (between 6.0 and 9.0 ppm). This is characteristic of α-helical structures.

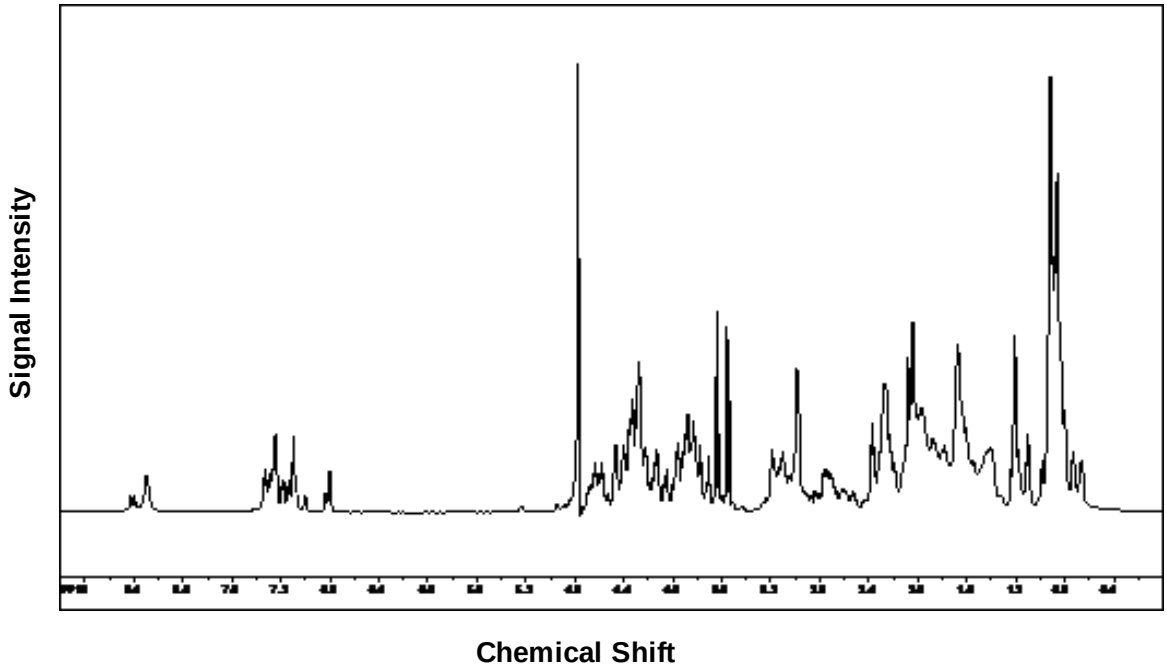


Figure 3.11 1H-NMR spectrum of unlabelled Snu114 1-122 at 25 °C. Figure illustrates 600 MHz 1H-NMR spectrum of unlabelled, isotopically normal Snu114 1-122 (0.7 mM) in 100% $2H_2O$, containing 50 mM sodium phosphate pH 6.2, 100 mM sodium chloride at 25°C. This experiment relvealed a lack of slowly exchanging labile NH protons (between 5.5 and 8.5 ppm). This indicates that there are limited tertiary interactions within Snu114 1-122. A CH shift peak at 5.2 ppm shows that the Snu114 1-122 is not denatured.

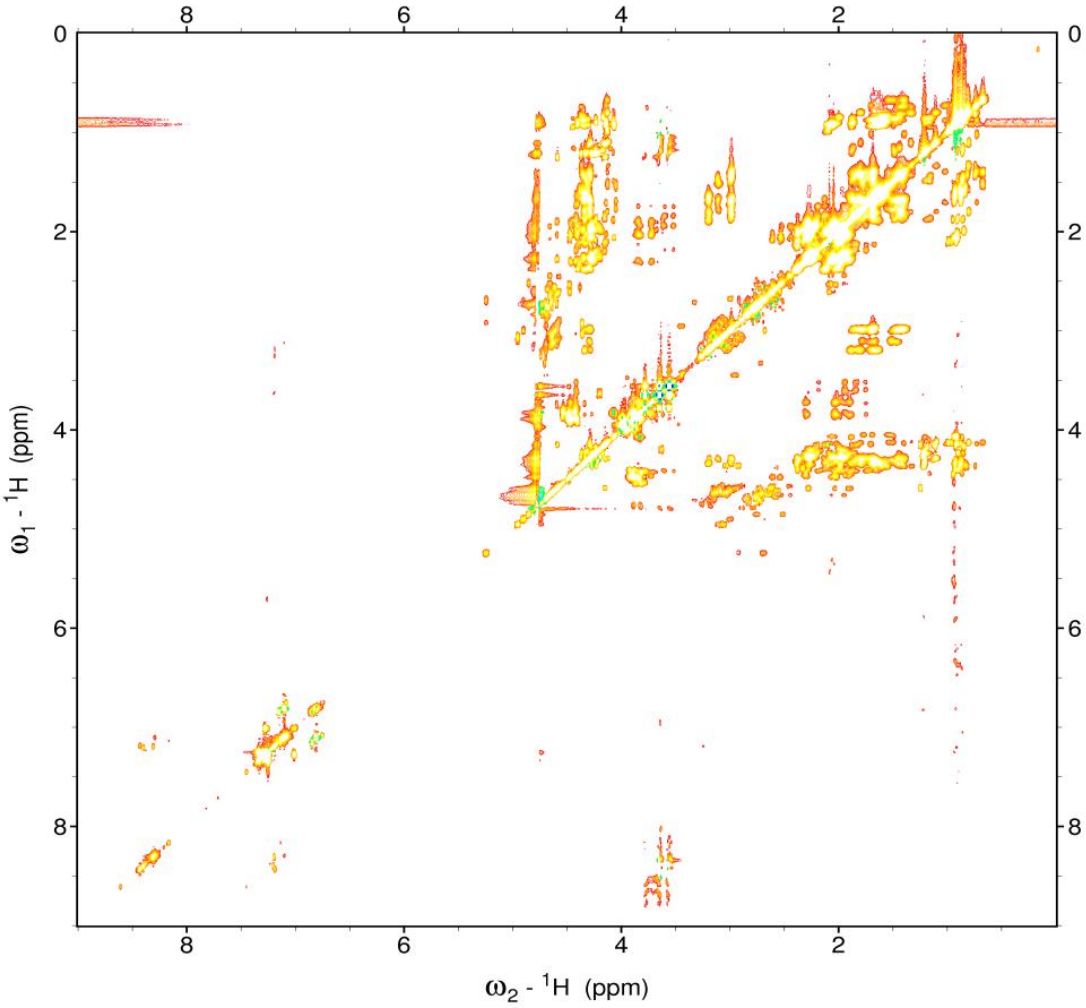


Figure 3.12 1H-NMR TOCSY spectrum of unlabelled Snu114 1-122 at 25 °C. Figure illustrates 600 MHz 1H-NMR TOCSY (mixing time=75 ms) spectrum of unlabelled, isotopically normal Snu114 1-122 (0.7 mM) in 100% $2H_2O$, containing 50 mM sodium phosphate pH 6.2, 100 mM sodium chloride at 25°C. . This experiment relvealed a lack of slowly exchanging labile NH protons (between 5.5 and 8.5 ppm). This indicates that there are limited tertiary interactions within Snu114 1-122. A CH shift peak at 5.2 ppm (seen as a shit along both axis from 5.2 ppm to around 2.8 ppm) shows that the Snu114 1-122 is not denatured.

which again suggests that there are limited tertiary interactions within the protein fragment. There was a single shifted CH proton at around 5.2 ppm which gave long range through bond connectivities in the TOCSY spectrum.

In summary the NMR analysis of unlabelled or isotopically normal Snu114 1-122 indicate that Snu114 1-122 is stable, but contains very little secondary structure. However, evidence suggests the possible presence of an alpha helical structure within Snu114 1-122. The presence of a shifted CH proton, seen in figure 3.12, demonstrates that Snu114 1-122 is not denatured.

3.1.4.4 Expression of uniformly ^{15}N labelled Snu114 1-122 for NMR studies

To enable more detailed NMR analysis of Snu114 1-122, uniformly ^{15}N labelled protein was produced. Incorporation of ^{15}N isotope into a protein allows information on the protein backbone to be collected via NMR. For the expression and purification of Snu114 1-122 for NMR, bacteria were grown in PASM-5052 media (Studier, 2005). This media was used as it allows labelling the protein fragment with ^{15}N, by replacing the nitrogen source in the media with $^{15}NH_4Cl$. Bacterial cells were grown and protein was purified in the same way as with the unlabeled Snu114 1-122. Purification steps were analysed by electrophoresis (Figure 3.13). ^{15}N labelled Snu114 1-122 was successfully purified and is in the same size range as the unlabelled Snu114 1-122 expressed for crystallography trials (Figure 3.13, lanes 4 to 9). Purified ^{15}N labelled Snu114 1-122 was dialysed, pooled and concentrated to give the required protein concentration for NMR, 10 mg/ml.

To allow more detailed information to be collected on Snu114 1-122, taking into account the bonds between nitrogen and hydrogen atoms, Snu114 1-122 had to be at least 95 % enriched with ^{15}N. Mass spectrometry was carried to check the ^{15}N enrichment via a

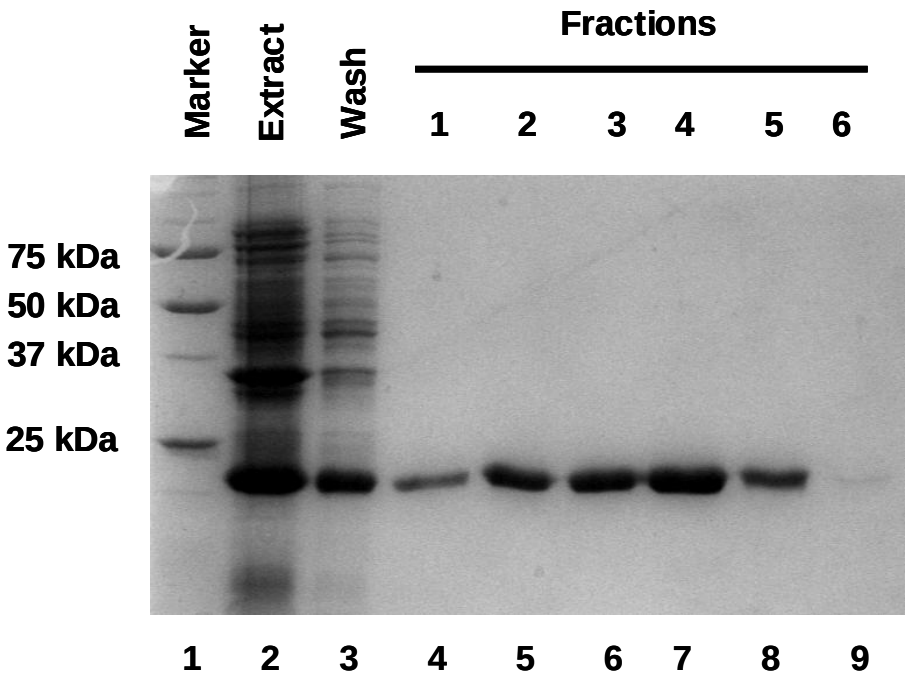


Figure 3.13 Purification of ^{15}N labelled Snu114 1-122. Coomassie blue stained 12% SDS-PAGE gel illustrating the steps in purification of ^{15}N labelled, N-terminally 6His-tagged Snu114 1-122 from bacteria. Snu114 1-122 was expressed in BL21 (DE3) cells grown in PASM 5052 media containing $^{15}NH_4Cl$ at 37°C for 36 hours. Bacterial extract was produced (lane 2), and the extract was applied to a HIS-Select Nickel affinity column. Wash from the column purification was analysed (lane 3). Purified protein was eluted with 250 mM imidazole and collected in 500 µl fractions (lanes 4 to 9). Lane 1 contains molecular weight marker.

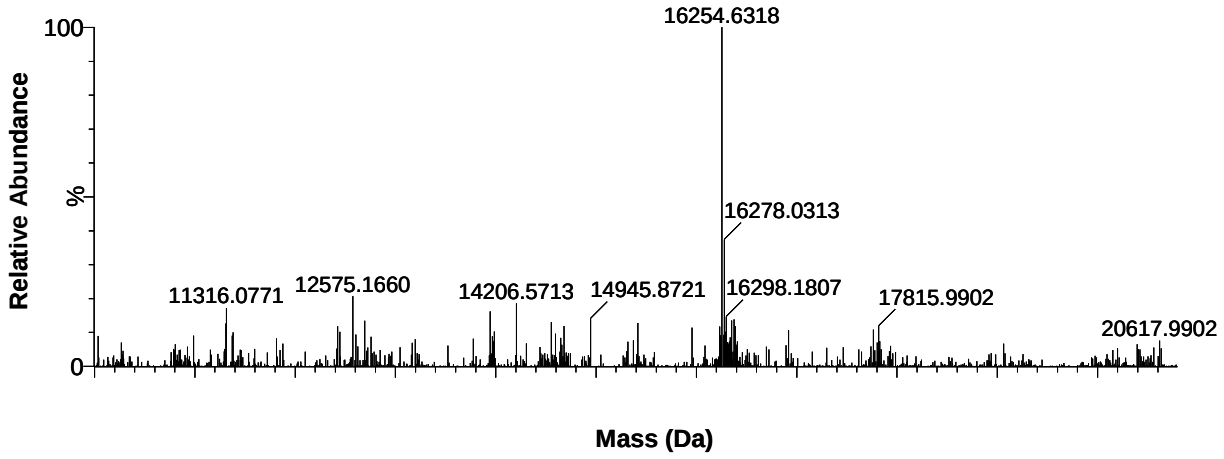


Figure 3.14 Mass spectrometry to determine the ^{15}N enrichment of labelled Snu114 1-122. To gain an accurate measurement of mass of ^{15}N labelled Snu114 1-122, mass spectrometry was carried out on a sample of purified then dialysed, ^{15}N labelled, 6His-tagged Snu114 1-122 at 50 pmol/µl in 10mM Tris-HCl, pH 7.4, 150 mM NaCl. This mass of the labelled protein was compared to the mass of unlabelled Snu1141-122 to determine the percentage of ^{15}N enrichment. The X axis represents the mass of protein detected in Da (also displayed at the top of each peak). The Y axis is a measure of the presence of all products detected by mass spectrometry, with the most abundant protein being scored as 100% and the amounts of all other products present being expressed as a percentage of that.

difference in mass between labelled and unlabelled protein (Figure 3.14). Labelled protein will have an increased mass due to incorporation of ^{15}N, rather than ^{14}N. Mass spectroscopy of ^{15}N labelled Snu114 1-122 revealed an apparent molecular mass of 16.255 kDa. Unlabelled Snu114 1-122 produced a mass of 16.061 kDa. This gives a mass difference of 0.194 kDa. There are 202 nitrogen atoms present in the Snu114 1-122 fragment, so a mass difference of 0.194 kDa means the protein fragment is 96 % enriched for ^{15}N. Therefore, high enough enrichment of ^{15}N was achieved to enable further analysis of Snu114 1-122 by NMR.

To collect further structural data regarding Snu114 1-122, 2D ^{15}N-Heteronuclear Single Quantum Coherence (HSQC) TOCSY was carried out on uniformly ^{15}N labelled Snu114 1-122, at 10 °C with a mixing time of 80 ms (Figure 3.15 A). This type of spectral analysis provides data on the protein backbone, with the 2D spectra having one axis for ^{1}H (X) and one for ^{15}N (Y). The ^{15}N-HSQC TOCSY spectrum showed a large number of both direct and long range ^{15}N-1H correlated connectivities with good sensitivity. Even though there was severe overlap, around 125 of these correlations could be identified. The labile amide protons resonate over a small spectral range (Figure 3.15 B). This suggests the presence of an α-helical or rod like structure. The spectra produced good sensitivity and long range through-bond correlations. This helped to identify 9 out of the 11 expected glycine spin systems, which resonate in a distinct and characteristic region. To allow determination of the structure of Snu114 1-122, further NMR analysis will be required, including analysis of double labelled ^{15}N ^{13}C Snu114 1-122.

To summerise, NMR carried out using ^{15}N labelled Snu114 1-122 confirms the relatively unstructured nature of Snu114 1-122, and supports the possibility of the presence of an alpha helical structure within Snu114 1-122.

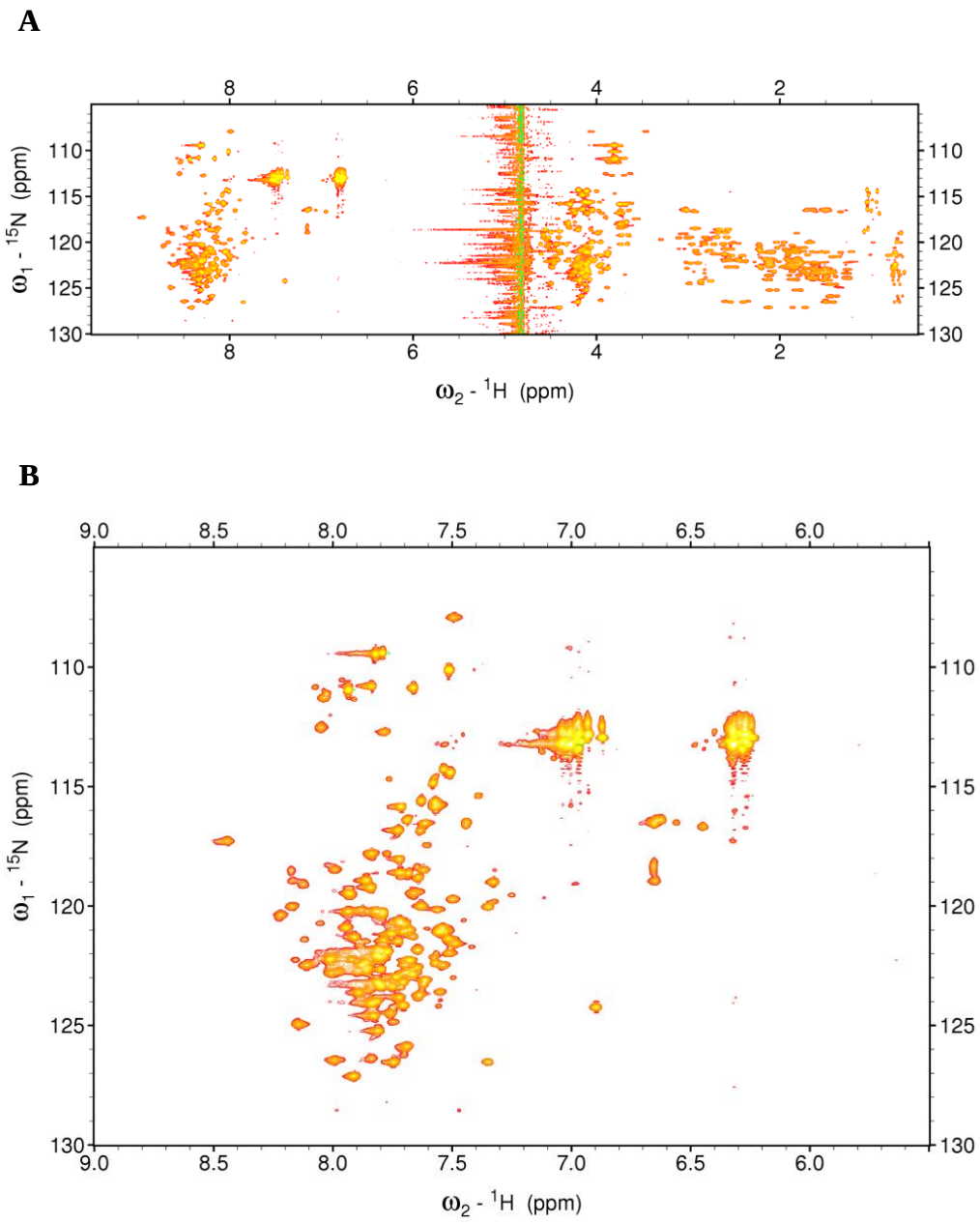


Figure 3.15 ^{15}N-HSQC TOCSY spectrum of uniformly ^{15}N labelled Snu114 1-122 at 10 °C. Panel A illustrates 600 MHz ^{15}N-HSQC-TOCSY spectrum of uniformly ^{15}N labelled Snu114 1-122 (0.7 mM) in 90% $1H_2O$+10% $2H_2O$, containing 50 mM sodium phosphate pH 6.2, 100 mM sodium chloride at 10°C measured with a mixing time of 80 ms. Panel B shows an enlarged view of the backbone amide region (5.5-9.0 ppm) of the spectrum shown in panel A. The cluster of spots present between 7.00-8.5 ppm on the x-axis and 112-130 on the y-axis indicates that Snu114 1-122 is unstructured. 9 out the expected 11 glycine spin systems can be identified between 7.4- 8.2 ppm on the x-axis and 106-113 ppm on the y-axis.

3.1.5 Functional analysis of the N-terminal region of Snu114p

As outlined, there are still many unknowns surrounding the function of the N-terminus of Snu114p such as, is the N-terminus involved in protein or RNA interaction? And how does the N-terminal region effect U4/U6 unwinding? Functional analysis of Snu114 1-122 was carried out in an attempt to answer some of these questions.

3.1.5.1 Influence of Snu114 1-122 on pre-mRNA splicing *in vitro*

To detect any possible dominant negative effects on pre-mRNA splicing, *in vitro* splicing assays were carried out using yeast whole cell extracts, to investigate the effects of purified Snu114 1-122 on pre-mRNA splicing. To enable analysis of splicing a ^{32}P labelled pre-mRNA substrate was added to the splicing reactions, allowing the reaction intermediates and products to be visualised by autoradiography following electrophoresis in a denaturing gel (Figure 3.16). Splicing reactions were carried out, in the presence of increasing amounts of purified Snu114 1-122 (Figure 3.16, lanes 8 to 13). A set of control reactions were also carried out, in which buffer was added in the place of protein (Figure 3.16, lanes 2 to 7). As the amount of Snu114 1-112 added to the splicing assay increased, the amount of intron lariat-exon intermediate, released intron lariat and spliced mRNA in the assays decreased (Figure 3.16, lanes 8 to 13). This was not observed in the control assays (Figure 3.16, lanes 2 to 7), indicating that the effects on splicing are a result of the addition of the Snu114 1-112. This suggests that addition of the Snu114p N-terminal fragment causes a defect in the first step of splicing.

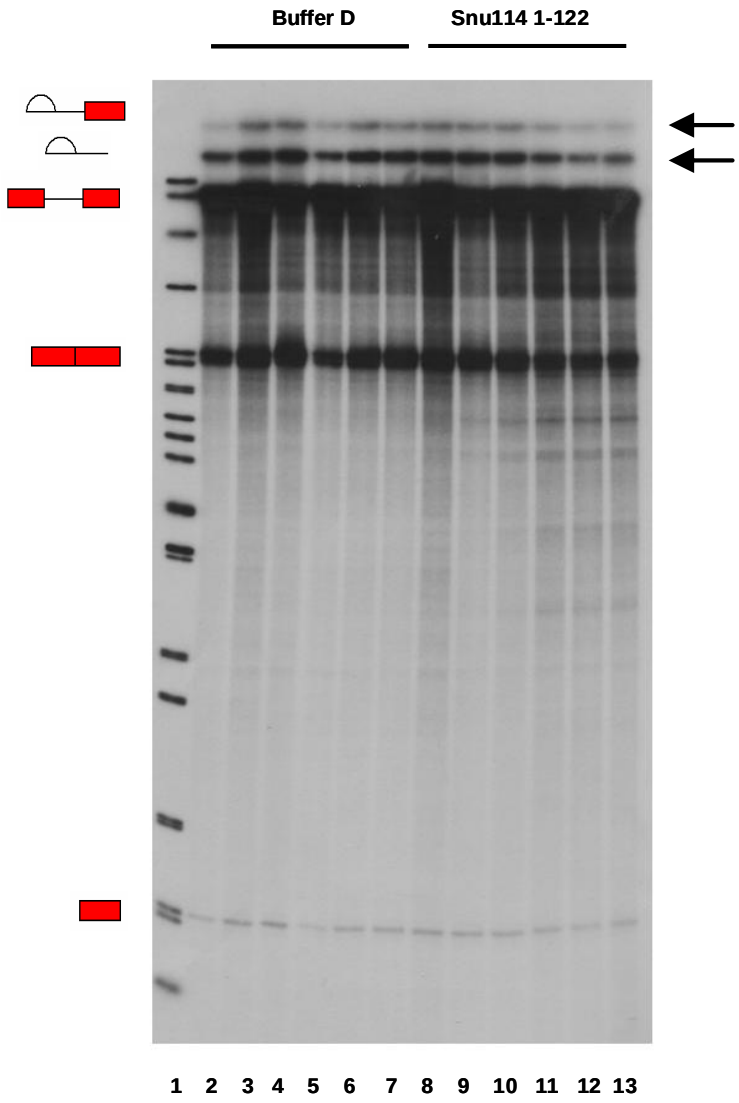


Figure 3.16 Analysis of *in vitro* pre-mRNA splicing in the presence of Snu114 1-122. Yeast whole cell extract was incubated under *in vitro* splicing conditions with ^{32}P body labelled *ACT1* pre-mRNA, and increasing amounts of Snu114 1-122 (lanes 8 to 13. 0, 3.96, 6.6, 9.24, 11.88 and 14.52 µg Snu114 1-122 respectively). A set of control reactions were carried out containing buffer D in the place of protein (lanes 2 to 7). RNA was isolated and analysed by denaturing PAGE. Pre-mRNA splicing reaction intermediates and products are indicated on the left. Lane 1 contains ^{32}P labelled pBR322 MspI-digested DNA. The black arrows indicate the positions of the intermediate produced following the first step of splicing and the released intron lariat. The amount of these two intermediates decreases with the addition of Snu114 1-122. This experiment was carried out ones and need to be repeated in triplicate to increase the validity of the results.

3.1.5.2 Analysis of snRNA binding properties of Snu114 1-122 *in vitro*

It has been shown that Snu114p can crosslink to U5 snRNA, and although the site of this interaction on U5 snRNA is known, it is still unknown which region of Snu114p is involved (Dix *et al*., 1998). To investigate whether the N-terminal region of Snu114p is involved in this interaction, or if it interacts with any of the other snRNAs of the spliceosome, electrophoretic mobility shift assays (EMSA) in native gels were carried out using the five snRNAs, U1, U2, U4, U5 and U6 (Figure 3.17). ^{32}P labelled U1, U2, U4, U5 and U6 snRNAs were each incubated with 0, 5 and 10 µM Snu114 1-122. Protein binding would be detected as a mobility shift of the snRNA band in the reactions containing protein. The addition of 5 µM or 10 µM protein did not result in a mobility shift with U1, U2, U4, U5 or U6 snRNAs. Therefore, under the conditions used in this experiment Snu114 1-122 does not bind any of the spliceosomal snRNAs *in vitro*.

3.1.5.3 Analysis of Snu114p N-terminal mutants *in vivo*

To allow investigations into any potential genetic interactions of the N-terminal region of Snu114p with the snRNAs of the spliceosome, and to try to identify any residues involved in these interactions, several Snu114p N-terminal mutants were constructed (Figure 3.18, A). Regions of conserved or highly polar amino acids were substituted for alanine residues, a non-polar and hydrophobic amino acid. Polar amino acids have side chains that tend to reside in an aqueous environment, so are usually found on the outer surface or exposed surface of proteins. Swapping polar amino acids for non-polar amino acids is predicted to disrupt the structure and/or function of a protein. Although leucine is more non-polar than alanine, the methyl group of alanine is non-reactive, so is rarely directly involved in protein function. For this reason swapping amino acids for alanine residues was expected to affect the function of the N-

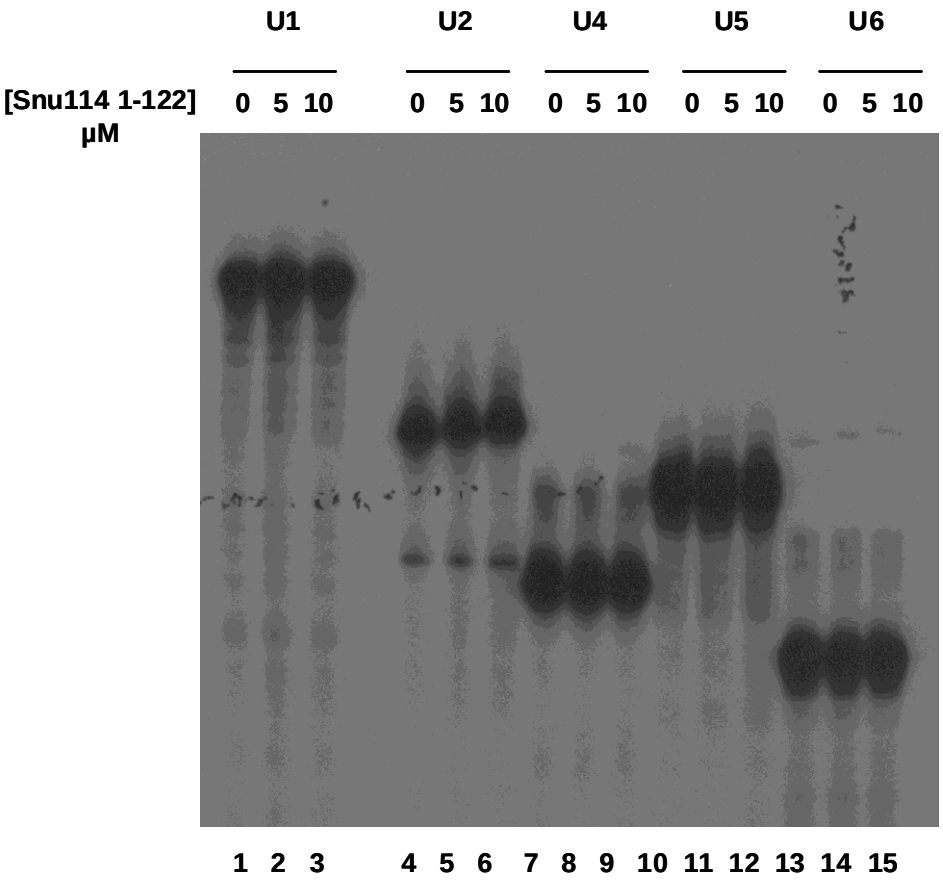


Figure 3.17 Electrophoretic mobility shift assays (EMSA) to investigate the binding of recombinant Snu114 1-122 with the snRNAs of the spliceosome. Native 5% acrylamide gel illustrating the results of EMSAs carried out containing Snu114 1-122 and one of each of the 5 snRNAs indicated above. ^{32}P end labelled snRNAs were incubated with either 0, 5 or 10 µM Snu114 1-122.

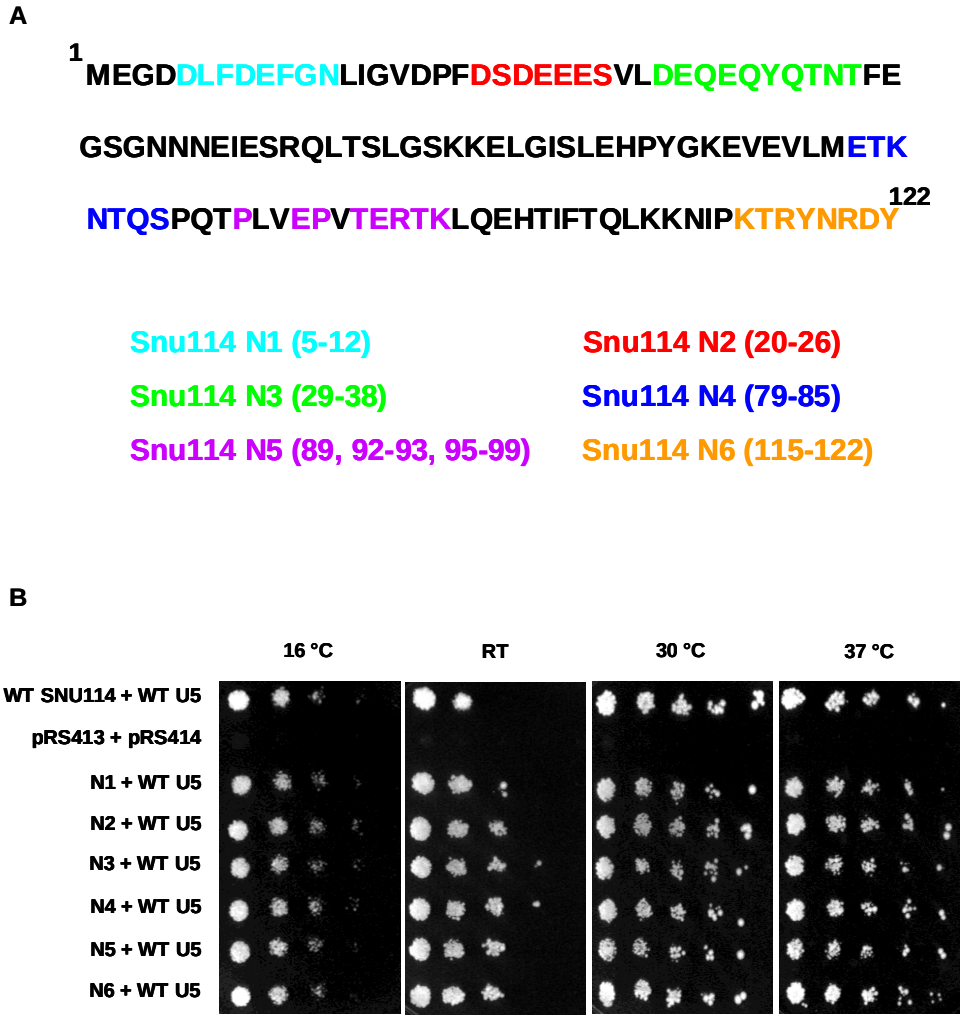


Figure 3.18 Six different Snu114 N-terminal mutants designed to investigate synthetic lethal interactions with snRNAs. A. Regions of conserved or highly polar amino acids (highlighted in colour) were all substituted for alanine residues, a non-polar and hydrophobic amino acid, in these mutants. The substituted regions are highlighted in the colour corresponding to that particular mutants name, seen in the same colour below. Amino acids substituted are indicated in brackets. B. The six Snu114 N-terminal mutants were tested for viability via a plasmid shuffle assay in the presence of wild-type U5. A 1 in 5 serial dilution was carried out starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. On each plate a positive control strain containing wild-type Snu114 and U5, and a negative control strain containing pRS413 and pRS414 were also present. Spot plates were incubated at 16°C, room temperature (RT), 30°C and 37°C.

terminal region of Snu114p.

Six *snu114* N-terminal mutants were constructed and tested for viability *in vivo* using a plasmid shuffle assay (Figure 3.19). Plasmid shuffle assays are carried out in haploid yeast strains with either one or two chromosomal genes knocked out. To complement the knocked out genes, wild-type copies of these genes are present in the yeast strain on a *URA3* plasmid. This yeast strain is transformed with mutant copies of the deleted genes, and colonies are transferred to media containing 5-flouroorotic acid (5FOA). Growth on 5FOA selects against the presence of the *URA3* plasmid containing the wild-type genes, leaving the mutant genes as the sole source of the gene or genes (Sikorski and Boeke, 1989). This determines if the mutant genes are sufficient to support growth, or whether the gene combination is lethal.

For analysis of the six *snu114* N-terminal mutants a haploid yeast strain with *SNU114* and the gene encoding U5 snRNA, *SNR7*, knocked out, and wild-type *SNU114* and U5 present on a *URA3* plasmid was utilised. This yeast strain was co transformed with one of the *snu114* mutants on a *HIS3* plasmid and wild-type U5 on a *TRP1* plasmid. Colonies containing mutant *snu114* and wild-type U5 were spotted onto 5FOA containing plates and incubated at 16°C, room temperature (RT), 30°C and 37°C to test for lethality and temperature sensitivity (Figure 3.18, B). A positive control strain containing wild type *SNU114* and wild-type U5 genes in pRS413 and pRRS414 vectors respectively, and a negative control containing pRS413 and pRS414 (that did not contain wild type *SNU114* or *SNR7*), were also spotted onto each plate. All of the mutants were viable at 16°C, RT, 30°C and 37°C. This indicates that the changes made to the N-terminal region do not have a major effect on the function of Snu114p.

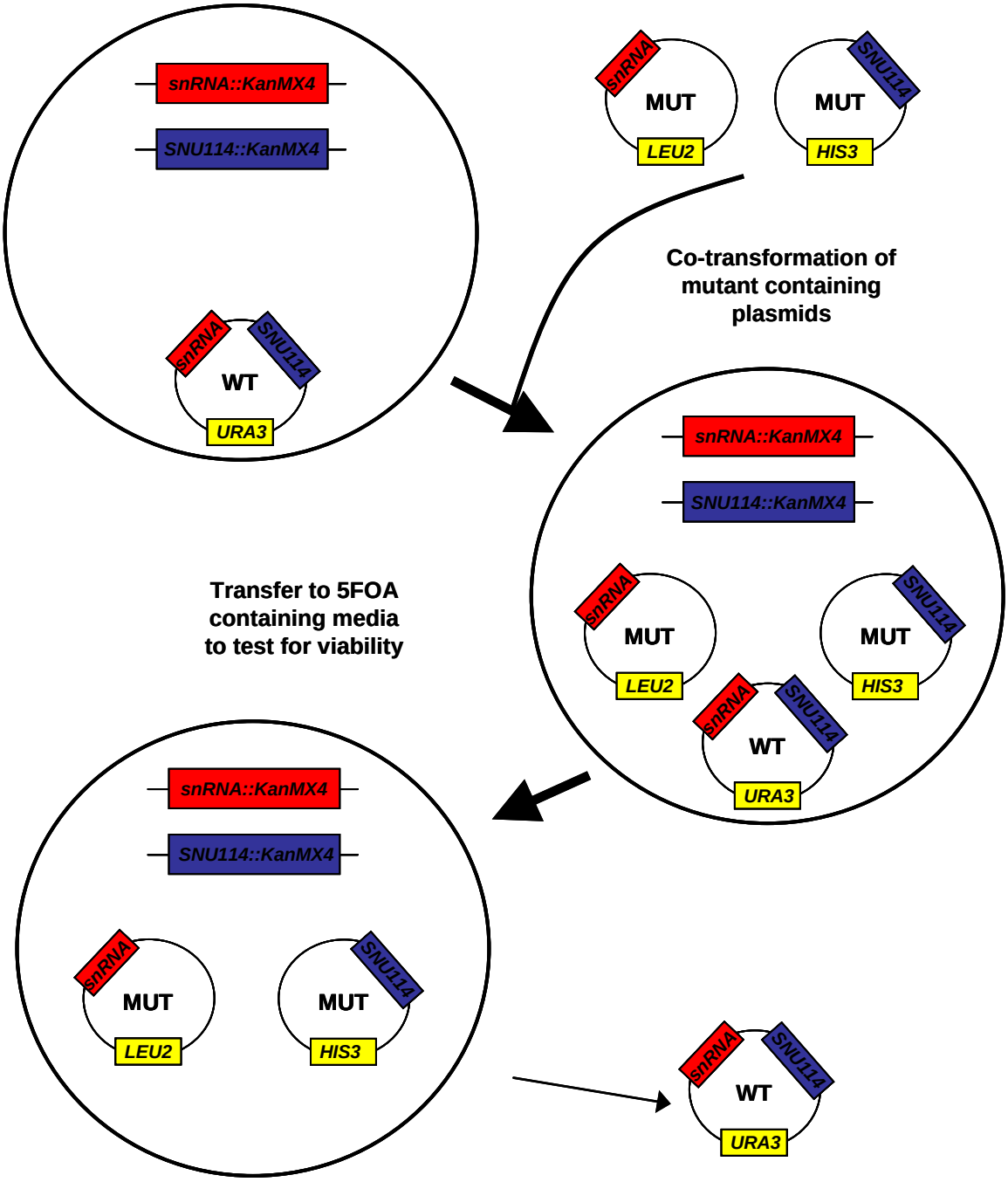


Figure 3.19 Illustration of the Plasmid Shuffle assay. A haploid yeast strain with the genes encoding Snu114p (*SNU114*) and an snRNA replaced with the KanMX4 marker is utilised for the plasmid shuffle assay. The *SNU114* and snRNA genes are complemented with wild-type (WT) copies of *SNU114* and snRNA on a single *URA3* plasmid. This strain is co-transformed with mutant copies (MUT) of *SNU114* on a *HIS3* plasmid and mutant copies (MUT) of an snRNA on a *LEU2* plasmid. Transformants are transferred to 5FOA containing media and the loss of the *URA3* plasmid is selected for, leaving the mutant genes as the sole source of *SNU114* and snRNA.

3.1.5.4 Investigating genetic interactions between Snu114p N-terminus and U5 snRNA *in vivo*

Snu114p N-terminal deletion (Snu114 ΔN) has been shown to be synthetically lethal with three U5 snRNA mutants, ΔC94,C95 and ΔU96,U97 which are deletions in the conserved loop 1, and ΔC112,G113 which is a deletion in the 3' side of the internal loop 1 (Figure 3.20, A) (Frazer *et al*., 2009). To test if the specific regions or residues of the Snu114p N-terminus involved in these interactions could be identified, a synthetic lethal screen was carried out. Firstly, the three U5 snRNA mutants that are synthetically lethal with Snu114 ΔN, were tested for viability in the Snu114/U5 knock out strain in the presence of wild-type *SNU114* at 16°C, RT, 30°C and 37°C (Figure 3.20, B). All the U5 snRNA mutants were viable at 16°C, RT and 30°C, but were sick at 37°C with very little growth at this temperature.

Each *snu114* mutant was tested at 16°C, RT, 30°C and 37°C with the three U5 snRNA mutants, giving 18 different combinations (Figure 3.21). All combinations of *snu114* mutants and U5 mutants were lethal at 37°C. This was expected as all of the U5 snRNA mutants are sick at 37°C in the presence of wild-type *SNU114*. Synthetic lethal interactions were identified between the U5 mutant ΔC112,G113 and the *snu114* mutants N1 and N6, with cells containing these mutations in combination failing to grow at any temperature tested. This indicates a genetic interaction between the 3' side of IL1 of U5 snRNA and Snu114p between amino acids 5 to 12 and 115 to 122. There was also a synthetic lethal interaction seen at 16°C between the *snu114* mutants N2 and N3, and the U5 snRNA mutant ΔC112,G113. Yeast containing these particular combinations were viable at 30°C, sick at RT, and failed to grow at 16°C. This would indicate that amino acids 20 to 26 and 29 to 38 of Snu114p are also involved in an interaction with the 3' side of IL1 of U5 snRNA.

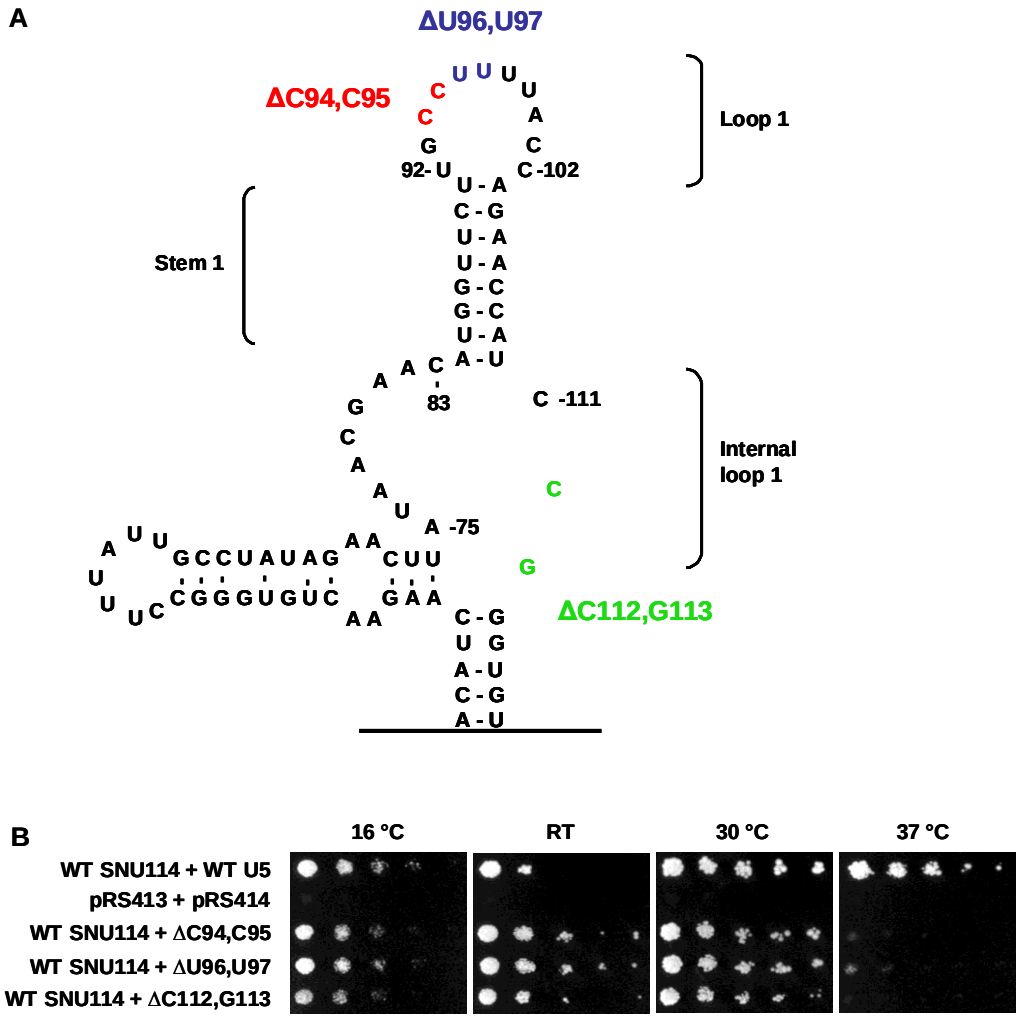


Figure 3.20 ***In vivo*** **analysis of three U5 snRNA mutants to be used a in synthetic lethal screen.** A. Three U5 snRNA mutants are known to be lethal with Snu114 ΔN. These mutations are highlighted on the diagram of the upper section of U5 snRNA, including Loop 1, Stem 1 and Internal Loop 1. B. The three U5 snRNA mutants were tested for viability in the presence of wild-type *SNU114*. A 1 in 5 serial dilution was carried out starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. On each plate a positive control strain containing wild-type *SNU114* and U5, and a negative control strain containing pRS413 and pRS414 were also present. Spot plates were incubated at 16°C, room temperature (RT), 30°C and 37°C.

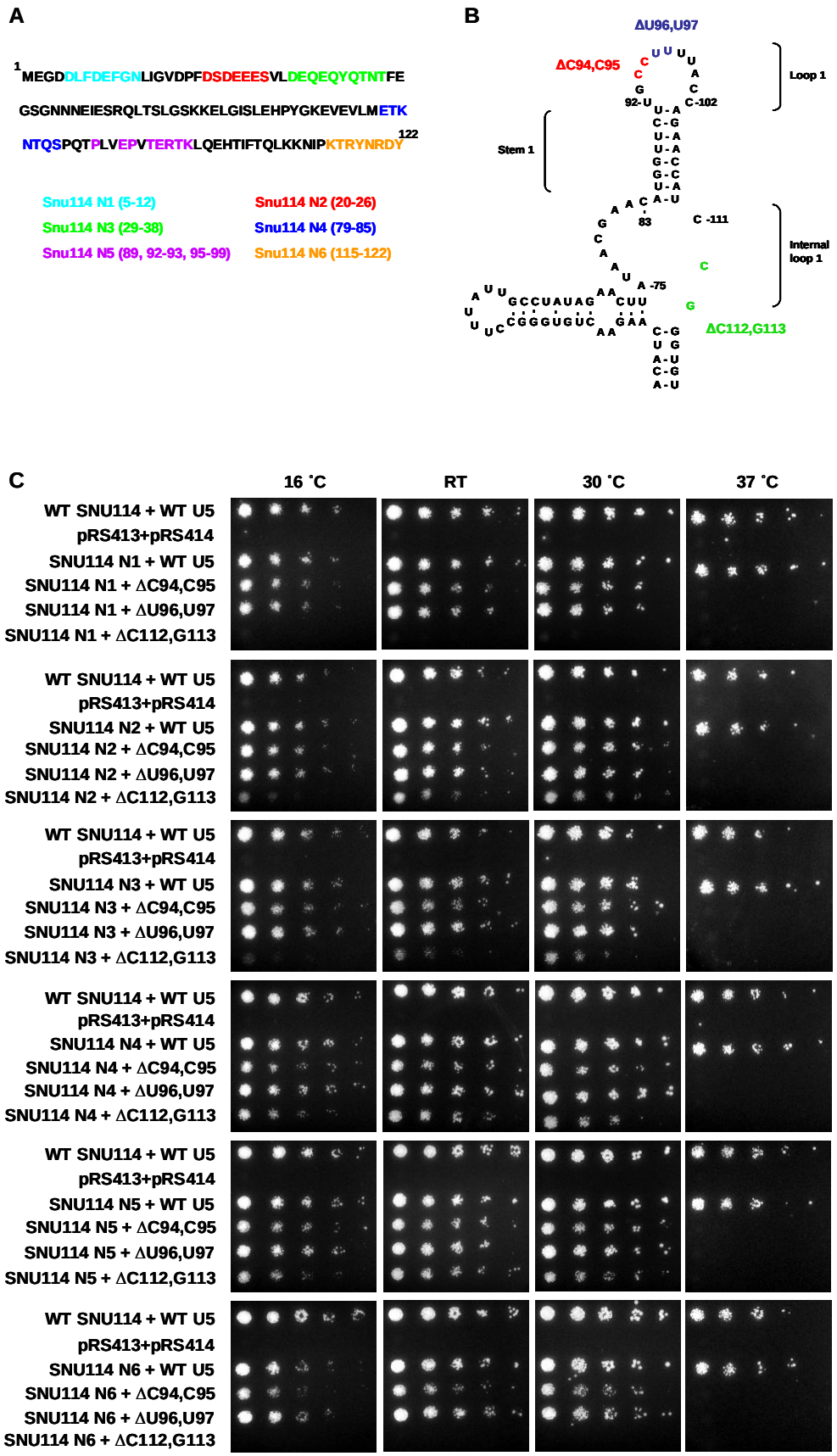

A
1MEGDDLFDEFGNLIGVDPFDSDEEESVLDEQEQYQTNTFE
GSGNNNEIESRQLTSLGSKKELGISLEHPYGKEVEVLMETK
NTQSPQTPLVEPVTERTKLQEHTIFTQLKKNIPKTRYNRDY122
Snu114 N1 (5-12)
Snu114 N2 (20-26)
Snu114 N3 (29-38)
Snu114 N4 (79-85)
Snu114 N5 (89, 92-93, 95-99)
Snu114 N6 (115-122)
B
ΔU96,U97
ΔC94,C95
Loop 1
Stem 1
Internal loop 1
ΔC112,G113
C
16 ˚C
RT
30 ˚C
37 ˚C
WT SNU114 + WT U5
pRS413+pRS414
SNU114 N1 + WT U5
SNU114 N1 + ΔC94,C95
SNU114 N1 + ΔU96,U97
SNU114 N1 + ΔC112,G113
WT SNU114 + WT U5
pRS413+pRS414
SNU114 N2 + WT U5
SNU114 N2 + ΔC94,C95
SNU114 N2 + ΔU96,U97
SNU114 N2 + ΔC112,G113
WT SNU114 + WT U5
pRS413+pRS414
SNU114 N3 + WT U5
SNU114 N3 + ΔC94,C95
SNU114 N3 + ΔU96,U97
SNU114 N3 + ΔC112,G113
WT SNU114 + WT U5
pRS413+pRS414
SNU114 N4 + WT U5
SNU114 N4 + ΔC94,C95
SNU114 N4 + ΔU96,U97
SNU114 N4 + ΔC112,G113
WT SNU114 + WT U5
pRS413+pRS414
SNU114 N5 + WT U5
SNU114 N5 + ΔC94,C95
SNU114 N5 + ΔU96,U97
SNU114 N5 + ΔC112,G113
WT SNU114 + WT U5
pRS413+pRS414
SNU114 N6 + WT U5
SNU114 N6 + ΔC94,C95
SNU114 N6 + ΔU96,U97
SNU114 N6 + ΔC112,G113

Figure 3.21 Plasmid shuffle assay to test for synthetic lethal interactions between six Snu114 N-terminal mutants and three U5 snRNA mutants. Six Snu114 N-terminal mutants (A) were tested for synthetic lethal interactions with three U5 snRNA mutants (B), via plasmid shuffle assays (C). The assay was carried in a *SNU114*/U5 knock out strain, which was co transformed with different combinations of *SNU114* and U5 mutants. A 1 in 5 serial dilution was carried out, starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. A positive control strain containing wild type *SNU114* and U5, and a negative control strain containing pRS413 and pRS414 were also spotted onto each plate. Spot plates were incubated at 16 °C, room temperature (RT), 30 °C and 37 °C

3.2 Discussion

Snu114p is an essential spliceosome component, involved in assembly and activation of the spliceosome, formation of the U5 snRNP and U4/U6 unwinding (Bartels et al., 2002; Bartels et al., 2003). Snu114p also functions in controlling disassembly of the post-splicing complex (Small et al., 2006). Snu114p has been identified as the only GTPase present in the spliceosome (Fabrizio et al., 1997). Although some roles of Snu114p have been identified, there are still many unanswered questions surrounding the exact mechanisms of actions and interactions of Snu114p. The expression and purification of three specific fragments of Snu114p was attempted to allow structural studies of Snu114p, and to allow *in vitro* functional analysis to be carried out. Where recombinant protein production was successful structural analysis of the fragment was attempted. *In vitro* splicing assays and *in vitro* binding studies using recombinant protein fragments were also carried out. *In vivo* analysis of *snu114* N-terminal mutants determined a synthetic lethal interaction between specific amino acids in the N-terminus of Snu114p and the 3' side of IL1 of U5 snRNA.

3.2.1 Expression of recombinant Snu114p fragments

3.2.1.1 Snu114 833-1008

The expression and purification of 6His-tagged Snu114 833-1008 C-terminal fragment was achieved but the yield of purified protein was very low in comparison to the yield of Snu114 1-122, so no further analysis of Snu114 833-1008 was attempted (Figure 3.3, lanes 5 to 7). The low level of purified Snu114 833-1008 is most likely due to poor protein expression, or the fact that most of the protein being expressed could be insoluble or unstable, rather than the purification procedure being inefficient. If Snu114 833-1008 had been highly expressed a strong band of protein at 21.84 kDa would be present in the bacterial extract (Figure 3.3, lane

8). However there was no strong band at 21.84 kDa, indicating that Snu114 833-1008 was either, not highly expressed, insoluble or expressed but was highly unstable and degradation had occurred. To determine if Snu114 833-1008 was expressed but was insoluble, a sample of bacteria cell pellet following sonication and centrifugation could have been analysed by SDS-PAGE. Insoluble protein would be present in the pellet and not the bacterial extract. To optimise expression of Snu114 833-1008, expression could be attempted with a different tag, such as maltose binding protein or a solubility-enhancement tag (SET) to attempt to increase the stability or solubility of Snu114 833-1008 (Kapust and Waugh, 1999; Zhou et al., 2001).

3.2.1.2 Snu114 1-400

Expression and purification of 6His-tagged Snu114 1-400 was attempted but protein of the expected size was not purified (Figure 3.4). As with Snu114 833-1008, the most likely reason for the failed purification, is that expression of Snu114 1-400 was very poor or that expressed Snu114 1-400 was insoluble or unstable. If 6His-tagged Snu114 1-400 was stable, insoluble and highly expressed, a band of highly expressed protein at 46.86 or 47.75 kDa would be seen in bacterial extracts (Figure 3.3, lanes 2 and 6 respectively). However, highly expressed Snu114 1-400 was not seen in either bacterial extract, indicating that either expression of Snu114 1-400 was poor, or that expressed Snu114 1-400 was insoluble or unstable so was present in the cell pellet or has degraded. As with Snu114 833-1008, to determine if Snu114 1-400 was expressed but was insoluble, a sample of bacterial cell pellet could be analysed via SDS-PAGE and Coomassie blue staining. It is possible that for stability of larger fragments of Snu114p, complete Snu114p needs to be present, to allow proper protein folding and allow it to take its natural conformation. However, published data on the purification of Snu114p from yeast cells identified break down products of around 75 and 50 kDa, found during purification

of the whole protein (Bartels et al., 2003). This indicates that Snu114p is not a very stable protein even when purified from yeast. To identify fragments of Snu114p that are expressed as stable protein fragments, mass spectrometry could be carried out to identify the 75 and 50 kDa breakdown products present in the purification of Snu114p from yeast. These fragments may be a product of instability at the weakest points within Snu114p, and may be stable themselves. To enable purification of Snu114 1-400, expression with different tags such as the maltose binding protein or solubility-enhancement tags (SET) could be attempted to improve the solubility and expression of Snu114 1-400 (Kapust and Waugh, 1999; Zhou et al., 2001).

Instability of protein fragments is often due to the amino acids present at the N-terminal end of the protein. It should be noted that both Snu114 833-1008 and 1-400 fragments have methionine at their N-termini. According to the N-end rule, methionine N-terminal residues provide the most stable N-termini, so any instability of Snu114 1-400 or Snu114 833-1008 is not due to the N-end rule (Tobias et al., 1991). In both cases outlined above several methods of improving expression and stability of the protein fragments, aside from the obvious choice of expressing fragments with a different tag, could be attempted. It is possible, particularly in the expression of larger fragments or proteins, that proteins are unable to reach their native conformation when expressed in bacteria, possibly effecting stability of the protein. To try and overcome this problem, molecular chaperones can be co-expressed in bacteria to aid protein folding (Baneyx, 1999; Baneyx and Mujacic, 2004). Also mRNA can be unstable in bacteria, so to increase protein production mRNA can be stabilized. This can be achieved in some mRNAs by having a stable secondary structure in the 5’ UTR (Baneyx, 1999). It also may be beneficial to try the expression at lower temperatures, as this can aid protein folding, solubility and prevent protein aggregation.

There is also a possibility that the stability of Snu114p and/or fragments of Snu114p may be effected by, or rely upon the presence of another protein or RNA, possibly Prp8p, Brr2p or U5 snRNA. Certain Snu114p mutants result in decreased association of Snu114p with Prp8p, Brr2p and U5 snRNA, and reduced levels of Prp8p (Brenner and Guthrie, 2006). When the interaction between Snu114p and Prp8p is destabilised, association of Brr2p with the U5 snRNA is also reduced (Brenner and Guthrie, 2006). As mutation of Snu114p effects its association with Prp8p, Brr2p and U5 snRNA and results in reduced levels of Prp8p, it should be considered that this reduction in Prp8p levels may be the result of induced instability of Prp8p (Brenner and Guthrie, 2006). This may indicate that the interaction between Snu114p, Brr2p and Prp8p affects the stability of these proteins and co-expression of Prp8p, Brr2p and possibly U5 snRNA may be beneficial for expression and purification of stable Snu114p, or fragments of Snu114p.

Another approach could be to overexpress the fragments in yeast. However this would not be possible in the case of Snu114 833-1008 as over expression of the C-terminus of Snu114p causes a growth defect in yeast (Akada et al., 1997). Expressing the fragment in yeast may aid stability of fragments as they will be in native conditions. Also, if any modifications or other proteins are required for proper protein folding and stability, they will be present in yeast, unlike in bacteria.

3.2.1.3 Snu114 1-122

In this study Snu114 1-122 has been cloned, expressed and purified in bacterial cells (Figure 3.5). 6His-tagged Snu114 1-122 was purified in concentrations high enough to carry out structural and functional investigations. The protein fragment did appear larger than expected when visualized on a 12% SDS-PAGE gel, so western blotting and mass spectrometry were

carried out to confirm that the purified protein fragment was 6His-tagged Snu114 1-122 (Figures 3.6 and 3.7). The protein purified from bacterial extract was detected by both the anti-His and anti-Snu114 antibodies by western blotting, indicating that the purified protein is the 6His-tagged N-terminal fragment of Snu114p (Figure 3.6). To confirm the size of 6His-tagged Snu114 1-122, mass spectrometry was carried out (Figure 3.7). The size of Snu114 1-112 expressed with an N-terminal 6His tag should be 16.19 kDa. Mass spectrometry revealed that the majority of protein present in the sample was 16.06 kDa, 0.13 kDa smaller than expected, although it appears larger on the SDS-PAGE gel. The smaller than expected size could mean that some degradation has occurred. The difference in mass is not large enough to account for the loss of a whole amino acid from either end of the fragment, with the N-terminal being Methionine (0.149 kDa) and the C-terminal amino acid being Tyrosine (0.181 kDa). It is possible that an amino acid side chain has been lost via degradation. The western blotting data and mass spectrometry do prove that the purified protein is His-tagged Snu114 1-122, therefore, Snu114 1-122 not migrating as expected on SDS-PAGE gel may be due to the highly acidic nature of Snu114 1-122 (Burton et al., 1981).

3.2.2 Structural analysis of Snu114 1-122

Determining the structure of a protein is advantageous in defining the function of a protein, as conclusions or hypothesis can be drawn from structural homology to proteins with known functions. The structure of all domains of Snu114p, excluding the N-terminus, have been predicted based upon homology with EF-2. Since there is no predicted structure of the N-terminus of Snu114p, solving the structure of Snu114 1-122 would be particularly interesting and novel. Attempts were made to solve the structure of Snu114 1-122, first via crystallography. X-ray diffraction revealed that all crystals formed during crystal growth trials

were in fact salt crystals (Figure 3.8, B and D), deeming the crystal growth trials unsuccessful. When initial crystal growth trials are unsuccessful several variables can be changed to promote crystal growth, including screening at different temperatures. Proteins can crystallise at a range of temperatures but in this study trials were only carried out at room temperature (Wiencek, 1999). Different well conditions could also be tried to promote crystal growth. Crystal trials were carried out using 384 different well conditions; however there are many different commercially available suites of conditions that could also be tested.

As crystal growth trials were unsuccessful, the alternative approach of NMR was attempted. NMR eliminates the need for protein crystal growth and just requires proteins to be in aqueous solution. 1D proton, 2D NOESY and 2D TOCSY NMR were carried out on unlabelled, isotopically normal Snu114 1-122. The 1D proton NMR indicated that Snu114 1-122 was stable, and contained little secondary structure (Figure 3.9). A 2D NOESY spectrum showed there were limited α-helical type amide-amide proton connectivities present in Snu114 1-122 (Figure 3.10). 1D proton and 2D TOCSY spectrum in 100% $^{2}H_2O$ indicated that there was very limited tertiary interactions within Snu114 1-122 (Figures 3.11 and 3.12). The presence of a single shifted CH proton showed that the protein fragment was not denatured (Figures 3.11 and 3.12).

For further analysis of 6His-tagged Snu114 1-122 via NMR, ^{15}N labelled Snu114 1-122 was required. This was achieved with 96% enrichment using PASM-5052 media and replacing the nitrogen source with $^{15}NH_4Cl$ (Figure 3.13) (Studier, 2005). ^{15}N enrichment was determined by detection of a mass difference between labelled and unlabelled Snu114 1-122 via mass spectrometry (Figures 3.7 and 3.14). 2D ^{15}N-HSQC TOCSY NMR was carried out on ^{15}N labelled Snu114 1-122 (Figure 3.15). This spectrum detected ^{15}N-^{1}H connectivities, with 125 being identified. 9 out of the expected 11 glycine spin systems present in Snu114 1-

122 were also identified. This spectrum also suggests the presence of an α-helical or rod like structure in Snu114 1-122. Since α-helices have an important role in DNA and RNA binding, by forming DNA or RNA motifs such as helix-turn-helix and leucine zipper motifs, it may suggest a role in RNA or DNA binding for Snu114 1-122.

The limited tertiary structure of the N-terminus of Snu114p may suggest that this protein domain exists as a flexible unit. The function of this may be to detect the state of certain aspects of the spliceosome, for example the state of the U4/U6 helix. This domain may undergo structure formation in response to specific cues within the spliceosome and activate other proteins, for example Brr2p to orchestrate the timing of U4/U6 unwinding, or activate the G domain of Snu114p.

These data suggest that the N-terminal region of Snu114p contains limited tertiary structure. However, more extensive analysis by NMR would require double labelled Snu114 1-122 containing both ^{15}N and ^{13}C. Even if a structure of Snu114 1-122 was solved, it should be noted that the structure of Snu114 1-122 when in isolation may be different to the structure of the N-terminal region when the rest of Snu114p is present. As phosphorylation sites in this region have been identified in the N-terminus of Snu114p, it is possible that phosphorylation of this protein fragment may alter the structure, in a similar manner to the glycogen phosphorylase enzyme (Gruhler et al., 2005; Johnson and Barford, 1993). Glycogen phosphorylase is controlled by reversible phosphorylation, and undergoes major conformation changes upon phosphorylation, going from the inactive to active form (Johnson and Barford, 1993). If the N-terminus of Snu114p is involved in any interactions with protein or RNA, binding events may also affect the structure or even induce the formation of a more complex structure in Snu114 1-122.

3.2.3 *In vitro* analysis of Snu114 1-122.

As Snu114p and, in particular, the N-terminal region are important for pre-mRNA splicing, purified 6His-tagged Snu114 1-122 was added to *in vitro* splicing assays to look for any dominant negative effects in formation of the splicing intermediates and product (Figure 3.16). Addition of Snu114 1-122 to *in vitro* splicing assays resulted in a reduction in intron lariat-exon intermediate (Figure 3.16, lanes 8 to 13). This indicates a defect in the first step of splicing. This could be due to a problem with assembly or activation of the spliceosome. The effect seen was not drastic, suggesting that the N-terminus of Snu114p does not carry out any major function in pre-mRNA splicing in isolation, and the functions of the N-terminus of Snu114p most probably involve other regions of Snu114p. The defect in the first step of splicing indicates that there may be some dominant negative effect. It is possible that Snu114 1-122 binds to a protein or snRNA that is required for the first step of splicing. With an excess of Snu114 1-122 in the splicing assays, any proteins or RNAs that bind to Snu114 1-122 might be bound by the excess of Snu114 1-122, and will not being incorporated into the spliceosome as they would under normal conditions, leading to a reduction in splicing. The reduction and not total block in splicing, indicates that addition of Snu114 1-122 is reducing the efficiency of splicing, rather than preventing it. Further analysis would be required to determine why the reduction in splicing intermediate was observed. Native gel analysis could be carried out to not only look at the snRNAs present in the splicing assays, but also the complexes they are in, for example the tri-snRNP, the post splicing complex and the whole spliceosome. This would provide information on spliceosomal assembly and activation and detect any defects that may be occurring due to the presence of Snu114 1-122. The splicing assays should be repeated in triplicate and quantified by phosphorimaging, to increase the validity of the findings and allow comparisons of the relative amounts of splicing intermediates. An experiment also needs to be

carried out to rule out the possibility that the effects seen are due to the presence of the 6His tag. Splicing reactions should be carried out with the addition of either a 6His-tagged protein unrelated to splicing or the addition of 6His-tag expressed by its self, to ensure the presence of the tag does not affect splicing. Addition of a His-tagged protein unrelated to splicing would also determine whether the effects seen were due to the addition of a protein to the assays, not specifically addition of Snu114 1-122.

With a suggestion of a dominant negative effect from the *in vitro* splicing assays, it was decided to investigate if Snu114 1-122 has any RNA binding activities. It is known that Snu114p can crosslink to the 5' side of the internal loop 1 of U5 snRNA and displays genetic interactions between the N-terminus of Snu114p, and U5 and U6 snRNAs (Dix et al., 1998; Frazer et al., 2009). With this in mind EMSAs were carried out to determine if the N-terminus of Snu114p interacts directly with any snRNAs (Figure 3.17). Addition of 5 or 10 μM of Snu114 1-122 to reactions containing an snRNA did not identify any detectable interactions between the N-terminus of Snu114p and the U1, U2, U4, U5 or U6 snRNA under the *in vitro* conditions used in the assay. This does not necessarily rule out the possibility that Snu114 1-122 is capable of binding snRNAs. It may just be that the conditions used in this experiment are not conducive for Snu114 1-122 to bind the snRNAs. It is also a possibility that Snu114 1-122 may be involved in snRNA interaction, as suggested by previous work, but requires the rest of, or at least other domains of, Snu114p to be present (Dix et al., 1998; Frazer et al., 2009). In light of recent data identifying two phosphorylation sites in the N-terminal region of Snu114p, and data suggesting that Snu114p can be ubiquitinated, it cannot be ruled out that modification of this region may affect or even induce snRNA binding (Gruhler et al., 2005; Peng et al., 2003; Verma et al., 2002). It should also be considered that the presence of the 6His tag on such a small protein fragment may affect the function or natural activities of

Snu114 1-122. Many things can affect such binding events, for example pH, the presence of other proteins, folding of the protein or snRNA. The lack of detection of protein-snRNA interactions in this experiment *in vitro*, does not totally rule out any Snu114 1-122 interactions with snRNAs *in vivo*. Another way to detect protein-RNA interactions of Snu114 1-122 would be to incubate 6His-tagged Snu114 1-122 in whole cell yeast extracts, subject to UV irradiation, and immunoprecpitate Snu114 1-122. RNA cross linked to Snu114 1-122 would be subjected to primer extension to detect any interacting snRNAs. The region of Snu114p involved in interactions with U5 snRNA has not yet been mapped, so this method could also be used to identify the regions of Snu114p that U5 snRNA cross links to. The human Snu114p (U5-116 kDa) has been shown to crosslink to a hairpin inserted into pre-mRNA downstream of the branch point, prior to the second step of splicing (Liu et al., 1997), so it would be interesting to see if Snu114p in yeast also interacts with the pre-mRNA.

3.2.4 *In vivo* studies of Snu114 N-terminal mutants

In vivo analysis of six N-terminal mutants has revealed that the N-terminal region of Snu114p is resilient to amino acid substitutions. Of the six mutants designed and tested all were viable and showed no temperature sensitivity (Figure 3.20). A previous study has shown that deletion of Snu114p N-terminus (Snu114 ΔN) is lethal when in combination with three different U5 mutants (Frazer et al., 2009). To narrow down the region of the N-terminus of Snu114p involved in these interactions, a synthetic lethal screen was carried out testing each of the *snu114* N-terminal mutants with the three U5 mutants that were lethal with Snu114 ΔN. This screen revealed a synthetic lethal interaction between two *snu114* mutants (N1 and N6), and the U5 IL1 mutant ΔC112,G113. This indicates an interaction between the U5 IL1 3' side and amino acids 5 to 12 and 115 to 122 of Snu114p. The screen also revealed a synthetic sick

interaction between *snu114* mutants N2 and N3 with U5 ΔC112,G113 at 16 °C, indicating that amino acids 20 to 26 and 29 to 38 of Snu114p are also involved in the interaction with U5 IL1. Therefore, it is the 38 N-terminal amino acids and the last 6 amino acids of the N-terminal domain of Snu114p (amino acids 121 and 122 are actually part of domain I) that are involved in the interaction with the 3' side of U5 IL1.

The absence of the N-terminal region was shown to be lethal with two U5 loop 1 mutants, in a previous study (Frazer et al., 2009). None of the N-terminal mutants tested here were synthetic lethal or sick with those U5 snRNA loop 1 mutants, so the regions of N-terminal amino acids involved in any interaction with U5 loop 1 were not narrowed down. This may suggest that the N-terminal region itself does not interact with U5 loop 1, but its presence is required to aid or stabilise the interaction between other regions of Snu114p and the U5 snRNA loop 1. It is also possible that the shape of Snu114p that is required for U5 loop 1 binding is partially dependent on the presence of the N-terminal region, or at least part of it.

A potential role of the N-terminus of Snu114p still to be investigated, is involvement in protein-protein interactions. Several different approaches to study potential protein-protein interactions of Snu114 1-122 may be attempted. This could include pull downs of His-tagged Snu114 1-122. Snu114 1-122 would be incubated with whole cell yeast extract, and immunoprecipitated with either the His antibody or Snu114p antibody and any associated proteins would be analysed by western blotting or coomassie blue staining of SDS-PAGE gels, followed by mass spectrometry. It is also possible that the N-terminus of Snu114p is involved in intramolecular interactions within Snu114p. The N-terminal region may fold back, interacting with the rest of Snu114p, possible playing a role in controlling the GTP state of Snu114p. An intramolecular interaction between Snu114 1-122 and the rest of Snu114p could

be tested for by incubating Snu114 1-122 with whole cell yeast extract of Snu114 ΔN strain, and trying to detect an interaction between Snu114 1-122 and Snu114 ΔN.

The data presented here would suggest that the N-terminal region of Snu114p does not carry out any major role in pre-mRNA splicing in isolation. Although the region is important in pre-mRNA splicing, its function probably involves the rest of Snu114p or another protein (Bartels et al., 2002). The role of the N-terminus could be to stabilise interactions of, or stabilise the structure of the rest of Snu114p, allowing an event in pre-mRNA splicing to be carried out efficiently. It is known that the N-terminal region is involved in U4/U6 unwinding so the N-terminus could be involved in control of Brr2p, possibly by communicating conformational changes of Snu114p brought about by GTP binding or hydrolysis, to Brr2p (Bartels et al., 2002). It may also help to stabilise an interaction between Brr2p and U4/U6. This theory could be tested by investigating the interaction between Brr2p and U4 and U6 snRNAs, and determining if it is reduced in a Snu114 ΔN strain. This would be done by immunoprecipitation of Brr2p in wild-type and Snu114 ΔN strains, and subjecting any RNA bound to Brr2p to primer extension. Since both the absence of the N-terminus of Snu114p and GDP bound Snu114p both repress U4/U6 unwinding, it is possible that the N-terminus of Snu114p is involved in promoting the GTP bound state of Snu114p (Bartels et al., 2002; Small et al., 2006). This could be by acting as a guanine nucleotide exchange factor (GEF) itself, or interacting and communicating the GTP state of Snu114p to a GEF, or even by playing a role in repressing a GTPase activating protein (GAP). To test these theories experiments need to be carried out to investigate how the GTP state of Snu114p is affected by deletion of its N-terminus. One approach to determine this would be to test if the phenotypes of snu114 mutants predicted to effect GTP binding of Snu114p, such as R478E and D271N (Bartels et al., 2003),

are exacerbated by deletion of the N-terminus of Snu114p. It should also be tested if the GTP state of Snu114p is still detected by the rest of the spliceosome in the absence of the N-terminus of Snu114p. For example, does GDP-bound Snu114 ΔN repress U4/U6 unwinding and disassembly of the post splicing complex, and does GTP-bound Snu114 ΔN derepress U4/U6 unwinding and disassembly of the post splicing complex, at the permissive temperature of Snu114 ΔN (Small et al., 2006). Further defining the protein-protein interactions of Snu114p, via immunoprecipitations, will help to determine if another protein interacting with Snu114p is acting as GEF or GAP.

4 Investigating interactions of the major U5 snRNP proteins with the U5 snRNA

Brr2p, Snu114p and Prp8p are the major U5 snRNP proteins conserved from *Saccharomyces cerevisiae* to humans (Fabrizio et al., 1997; Lossky et al., 1987; Will et al., 1993; Xu et al., 1996). Snu114p is the only GTPase required for pre-mRNA splicing, and functions during spliceosome assembly, U4/U6 unwinding in spliceosomal activation and disassembly of the post splicing complex (Bartels et al., 2002; Bartels et al., 2003; Brenner and Guthrie, 2005, 2006; Fabrizio et al., 1997; Small et al., 2006). Brr2p is a DExH box protein which is involved in U4/U6 unwinding and disassembly of the post splicing complex, possibly via U2/U6 unwinding, and is thought to be controlled by the guanine nucleotide state of Snu114p (Raghunathan and Guthrie, 1998; Small et al., 2006; Xu et al., 1996). Prp8p is a large protein situated at the core of the spliceosome and is hypothesised to regulate major rearrangements and contribute directly to the active site (Abelson, 2008; Collins and Guthrie, 1999). The U5 snRNP is required for both steps of splicing, is present in the active spliceosome and aligns the exons at the catalytic core of the spliceosome to allow splicing to occur (Cheng and Abelson, 1987; Newman and Norman, 1991; Newman and Norman, 1992; O'Keefe and Newman, 1998; O'Keefe et al., 1996; Sontheimer and Steitz, 1993; Wyatt et al., 1992). Although it is well documented that Snu114p, Brr2p and Prp8p are all part of the U5 snRNP, little is known about the regions of U5 snRNA required for association of these proteins with the U5 snRNP, and if the requirements for association with U5 snRNA are different for Snu114p, Brr2p and Prp8p. Defining the requirements for association of Brr2p, Snu114p and Prp8p with the U5 snRNP will provide information on U5 snRNP assembly, whether Brr2p, Snu114p and Prp8p bind U5 snRNA independently and if different regions of U5 snRNA are required for association of each protein. This will further increase knowledge and understanding of the protein-RNA

interactions that occur within a major spliceosome component present at the active core of the spliceosome.

4.1 Results

4.1.1 Brr2p, Snu114p and Prp8p interact with U2, U4, U5 and U6 snRNAs

Protein-RNA interactions are essential in forming the catalytic core of the spliceosome, and although splicing is thought to be RNA catalysed, core proteins of the spliceosome are almost certainly required to arrange the RNAs in the correct orientation to allow splicing to occur. Brr2p, Snu114p and Prp8p are all known to be U5 snRNP components and Snu114p and Prp8p have both been shown to crosslink to the U5 snRNA (Dix et al., 1998). Prp8p has also been shown to crosslink to the U6 snRNA (Vidal et al., 1999). Brr2p, Snu114p and Prp8p function in U4/U6 unwinding and disassembly of the post splicing complex (Bartels et al., 2002; Bartels et al., 2003; Brenner and Guthrie, 2005, 2006; Raghunathan and Guthrie, 1998; Small et al., 2006; Xu et al., 1996). To investigate whether Brr2p, Snu114p and Prp8p associate with any other snRNAs while functioning in pre-mRNA splicing, and to confirm that U5 snRNA associates with these proteins in yeast whole cell extract, immunoprecipitations were done. Due to the lack of an anti-Brr2p antibody, Prp8p, Snu114p and Brr2p were TAP-tagged to enable immunoprecipitation of all three proteins using the same method. However, the addition of a TAP tag to the C-termius of Prp8p resulted in a slow growth phenotype (data not shown), so Prp8p was immunoprecipitated using anti-Prp8p antibodies. For analysis of the interactions of Brr2p and Snu114p with snRNAs of the spliceosome, yeast whole cell extracts were produced from strains containing TAP-tagged Brr2p or Snu114p. Brr2p and Snu114p were each immunoprecipitated and any RNA associated with the TAP-tagged protein was isolated. The isolated RNA was subjected to primer extension using primers specific to U1,

U2, U4, U5 and U6 snRNAs. As a control immunoprecipitations were performed using yeast whole cell extract from a strain containing TAP-tagged Prp21p. Prp21p is a U2 snRNP component and should immunoprecipitate U2 snRNA (Arenas and Abelson, 1993). Immunoprecipitations using yeast whole cell extract from a strain containing no tagged proteins were carried out as a negative control. To investigate the interactions of Prp8p with snRNAs an alternative approach was used. It is known that the introduction of a tag to Prp8p can affect its function (Boon et al., 2007; Maeder et al., 2009), so immunoprecipitations using anti-Prp8p antibody were performed, eliminating the need for tagging of Prp8p. RNA associated with the immunoprecipitated protein was purified and subjected to primer extension using primers specific to U1, U2, U4, U5 and U6 snRNAs. Negative control immunoprecipitations were done without adding yeast whole cell extract, and in the absence of Prp8 antibody. Total RNA extracted from each whole cell extract was tested via primer extension using primers specific to U1, U2, U4, U5 and U6 snRNAs, to confirm that these snRNAs were all present and detectable via primer extension. Western blotting was carried out to analyse the immunoprecipitated protein and confirm the correct protein was being immunoprecipitated in each experiment.

The immunoprecipitations performed demonstrate that Brr2p, Snu114p and Prp8p all associated with U2, U4, U5 and U6 snRNAs in yeast whole cell extracts (Figures 4.1, 4.2 and 4.3, lane 2). In the Brr2p and Snu114p experiments the Prp21p controls reveal that Prp21p only associates with U2 snRNA as expected (Figure 4.1 and 4.2, lanes 3 and 4). This was expected because Prp21p is present in the pre-spliceosome, and is tightly associated with the U2 snRNA, but not with the U1 snRNA, therefore Prp21p only immunoprecipitates U2 snRNA (Arenas and Abelson, 1993). The negative controls using extract from an untagged yeast strain did not immunoprecipitate any snRNAs (Figure 4.1 and 4.2, lanes 3 and 4). Primer

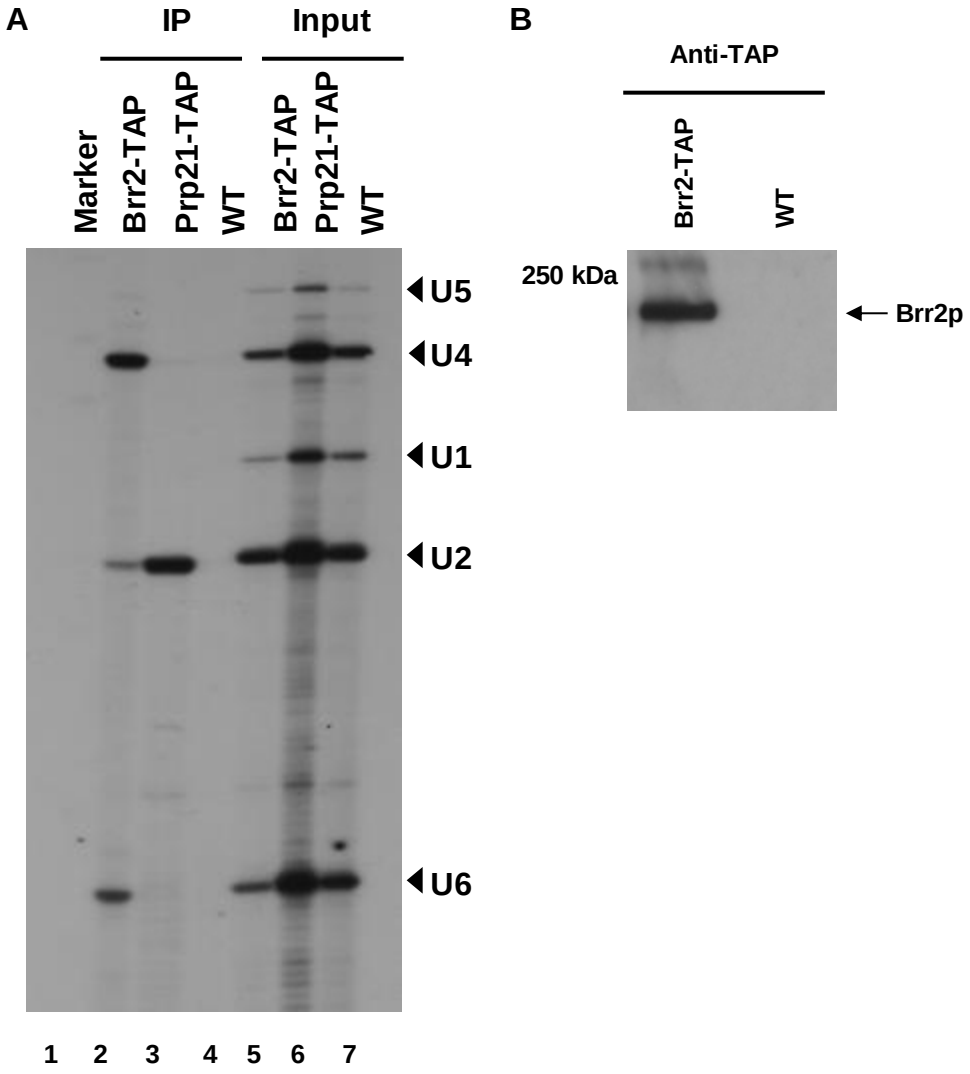


Figure 4.1 Brr2p associations with snRNAs in yeast whole cell extracts. A. Primer extension analysis of RNA isolated following immunoprecipitation (IP) of Brr2-TAP extract (lane 2), Prp21-TAP extract (lane 3) and an untagged extract (lane 4). Primers were specific for U1, U2, U4, U5 and U6 snRNAs as indicated on the right. Primer extension of total RNA isolated from each extract used was also performed (lanes 5 to 7). The products of primer extensions were separated by denaturing PAGE with ^{32}P labelled pBR322 MspI-digested DNA (lane 1). B. Brr2-TAP immunoprecipitation was confirmed by western blotting with anti-TAP antibody. The position of Brr2-TAP on the blot is indicated by the arrow on the right.

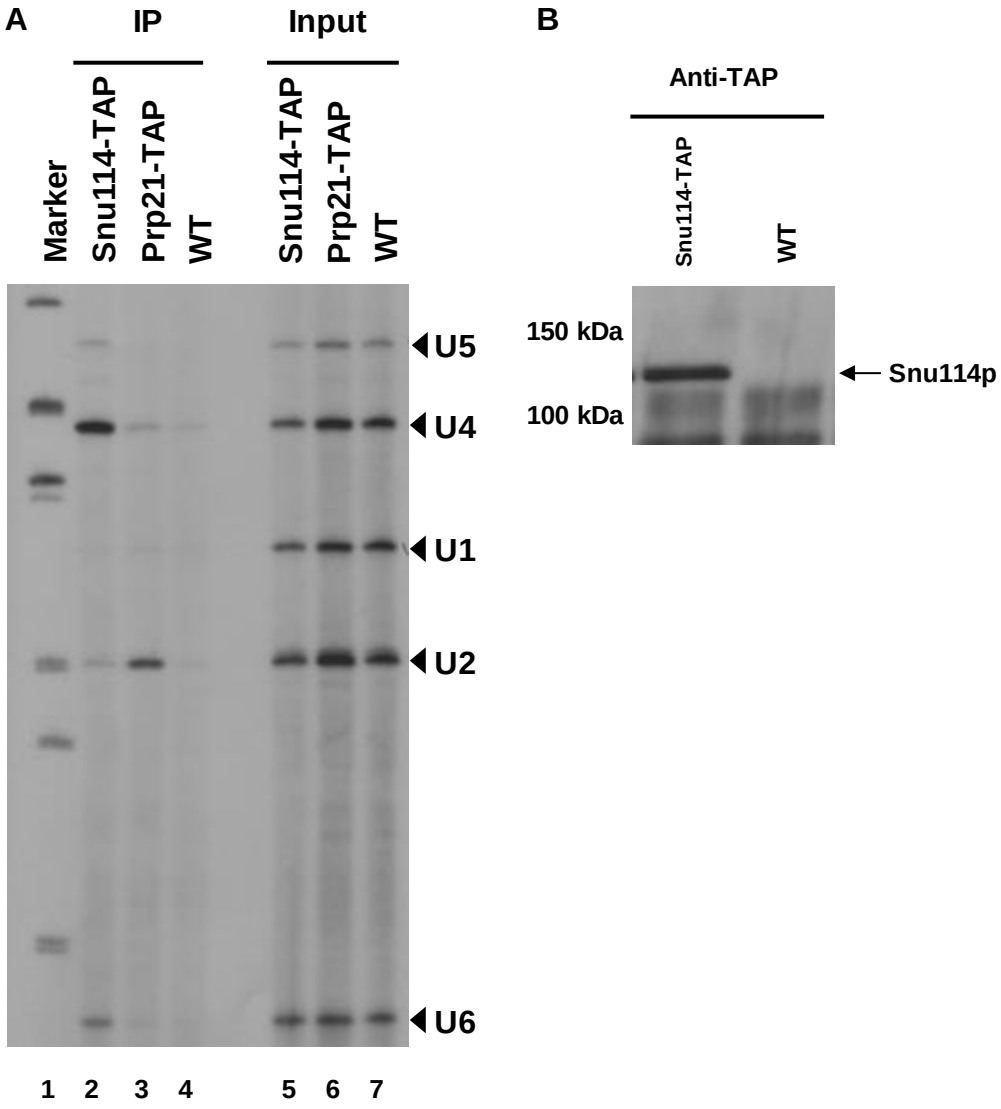


Figure 4.2 Snu114p associations with snRNAs in yeast whole cell extracts. A. Primer extension analysis of RNA isolated following immunoprecipitation (IP) of Snu114-TAP extract (lane 2), Prp21-TAP extract (lane 3) and an untagged extract (lane 4). Primers were specific for U1, U2, U4, U5 and U6 snRNAs as indicated on the right. Primer extension of total RNA isolated from each extract used was also performed (lanes 5 to 7). The products of primer extensions were separated by denaturing PAGE with ^{32}P labelled pBR322 MspI-digested DNA (lane 1). B. Snu114-TAP immunoprecipitation was confirmed by western blotting with anti-TAP antibody. The position of Snu114-TAP on the blot is indicated by the arrow on the right.

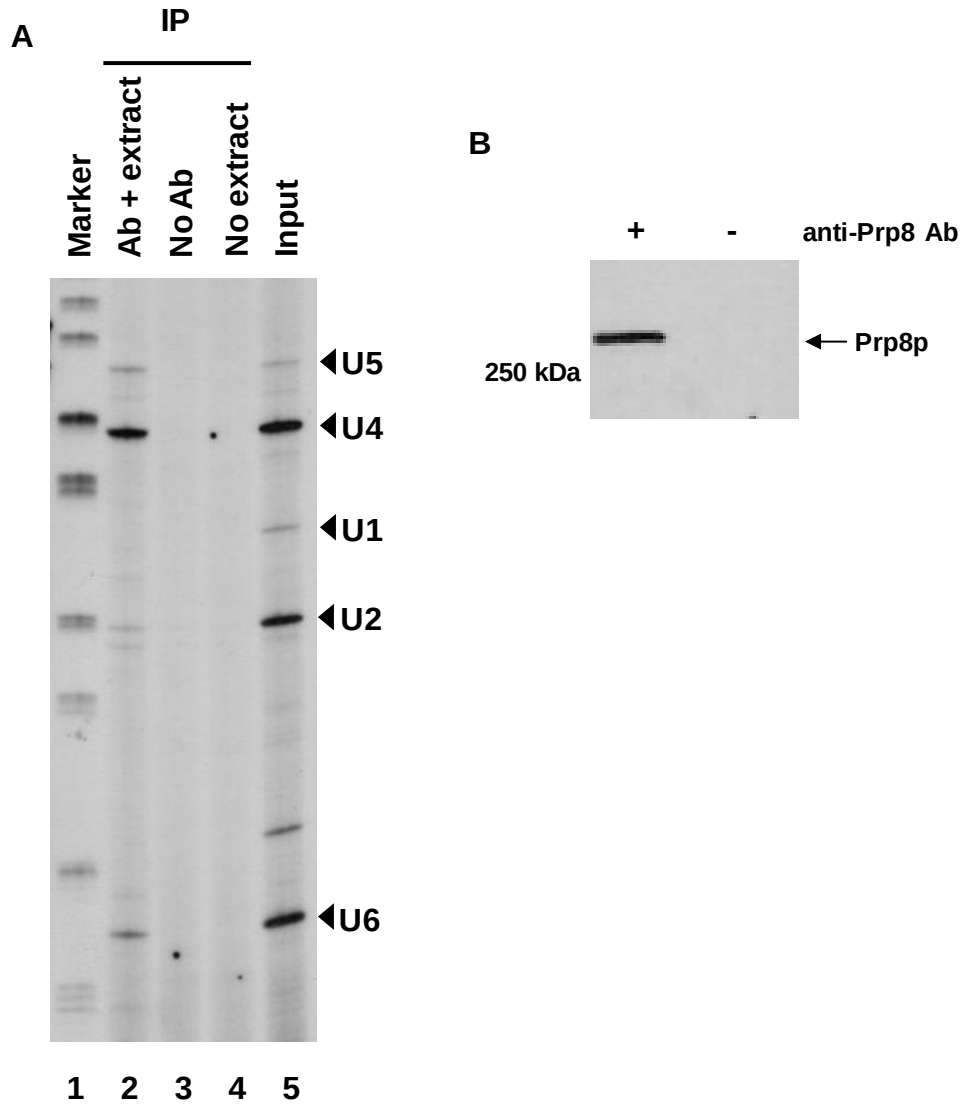


Figure 4.3 Prp8p associations with snRNAs in yeast whole cell extracts. A. Primer extension analysis of RNA isolated following immunoprecipitation (IP) with anti-Prp8p antibodies (Ab) from wild type extracts (lane 2). Negative control IPs using no antibody and no extract were performed (lanes 3 and 4). Primers were specific for U1, U2, U4, U5 and U6 snRNAs as indicated on the right. Primer extension of total RNA isolated from the extract used in IPs was also carried out (lane 5). The products of primer extensions were separated by denaturing PAGE with ^{32}P labelled pBR322 MspI-digested DNA (lane 1). B. Prp8p immunoprecipitation was confirmed by western blotting with anti-Prp8p antibody (Ab). The position of Prp8p on the blot is indicated by the arrow on the right.

extension of the negative controls in the Prp8p experiment did not detect the presence of any snRNAs, as expected (Figure 4.3, lanes 2 and 3). Western blotting of Brr2p and Snu114p immunoprecipitations with anti-TAP antibodies (Figures 4.1B and 4.2B, respectively), and Prp8p immunoprecipitations with anti-Prp8p antibodies (Figure 4.3B), confirmed in each case that the correct protein was immunoprecipitated.

4.1.2 U5 snRNA requirements for Brr2p, Snu114p and Prp8p association

It is already known that Brr2p, Snu114p and Prp8p all interact with the U5 snRNA; however, little is known about the regions and specific nucleotides required for association of these proteins with U5 snRNA. It has been published that Snu114p cross-links to a nucleotide in the 5' side of U5 snRNA Internal Loop 1 (IL1), and Prp8p cross-links to five regions of U5 snRNA (Dix et al., 1998). Genetic interactions between Snu114p and Loop 1 and IL1 of U5 snRNA have also been identified (Frazer et al., 2009). To further define the regions of U5 snRNA required for the association of Brr2p, Snu114p and Prp8p with U5 snRNA, several U5 snRNA mutants were constructed for use in immunoprecipitations. A reduction in association of a U5 snRNA mutant with a U5 snRNP protein, compared to wild-type U5 snRNA, would suggest that the mutated region is involved in the association of that U5 snRNP protein with the U5 snRNA.

4.1.2.1 Analysis of U5 snRNA mutants *in vivo*

Three regions of U5 snRNA were chosen for mutagenesis to allow investigations into the U5 snRNA requirements for Brr2p, Snu114p and Prp8p association with this snRNA (Figure 4.4). The first region chosen for mutagenesis was the 3' side of U5 snRNA IL1. The 3' side of IL1 was investigated as it is essential for U5 snRNA function and both Snu114p and Prp8p are

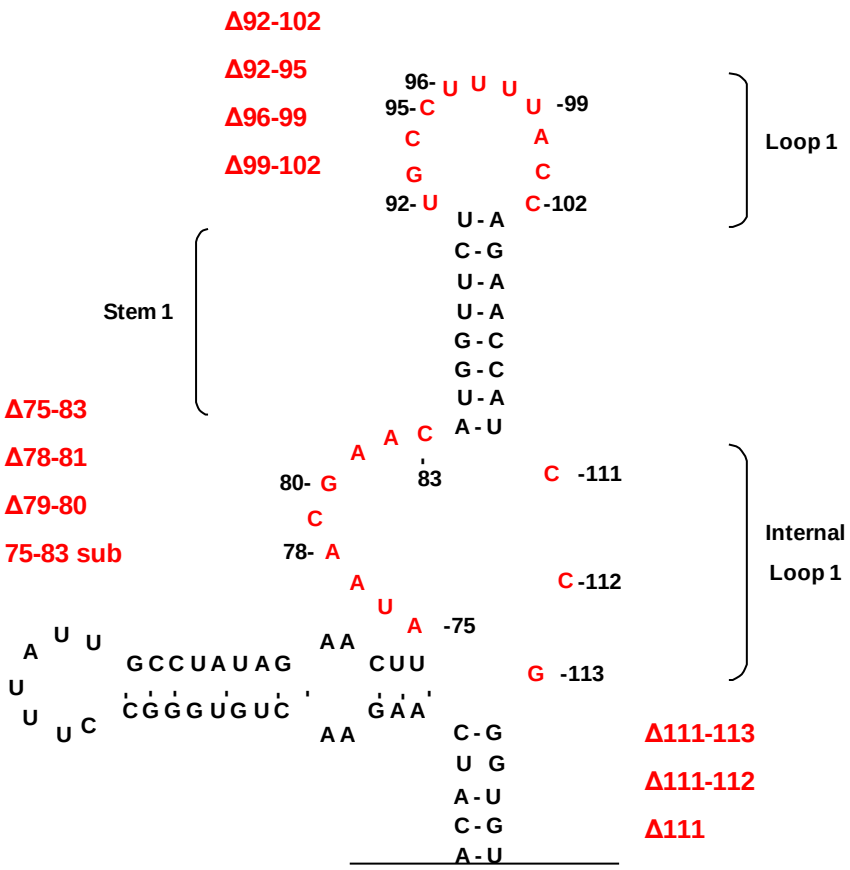


Figure 4.4 U5 snRNA mutants designed to investigate association of Brr2p, Snu114p and Prp8p. Three regions of U5 snRNA were mutated to investigate the effects of mutation on the association of U5 snRNP proteins. The three regions chosen for investigation were Loop 1 and the 3' and 5' sides of Internal Loop 1 (IL1). These regions are highlighted in red on this diagram of the upper section of U5 snRNA, and the mutations constructed are listed in red.

known to cross-link to position C79 in this region (Dix et al., 1998; Frank et al., 1994). The 5' side of U5 snRNA IL1 was also chosen for analysis as is conserved between humans and yeast, is required for U5 snRNA function and Prp8p cross-links to this region (Dix et al., 1998; Frank et al., 1994). In human U5 snRNA, both sides of IL1 (called IL2 in humans) are necessary for efficient expression of U5 snRNA, U5 snRNP formation and spliceosome assembly (Hinz et al., 1996). The third region chosen for analysis was Loop 1 of U5 snRNA. U5 Loop 1 was chosen due to it being highly conserved, essential for U5 snRNA function, having a vital role in aligning exons for splicing, and for being a site of Prp8p crosslinking (Dix et al., 1998; Frank et al., 1994; Newman and Norman, 1991; Newman and Norman, 1992; O'Keefe and Newman, 1998; O'Keefe et al., 1996). Studies on the human U5 snRNA have shown that Loop 1 is involved in human Prp8p binding (Hinz et al., 1996).

Four different mutations were constructed in the 5' side of U5 snRNA IL1 (nucleotides 75 to 83). The first mutation was deletion of the whole 5' side of IL1, nucleotides 75 to 83 (Δ75-83). The 5' side of U5 snRNA IL1 was also subjected to two smaller deletions, with nucleotides 78 to 81 (Δ78-81) and 79 to 80 (Δ79-80) being deleted. A final mutant was constructed in which nucleotides 75 to 83 in the 5' side of IL1 were substituted for the reverse complement of the wild-type sequence (75-83 sub) (Figure 4.4). Four deletions were made in Loop 1 of U5 snRNA, the first being deletion of the whole Loop, nucleotides 92-102 (Δ92-102). Loop 1 was also deleted in three smaller sections, nucleotides 92-95 (Δ92-95), 96-99 (Δ96-99) and 99-102 (Δ99-102) (Figure 4.4). Three mutants were constructed containing deletions in the 3' side of U5 snRNA IL1. The first mutation made in the 3' side of IL1 was deletion of nucleotides 111 to 113 (Δ111-113), the second being deletion of nucleotides 111 and 112 (Δ111-112) and the final mutation being a single nucleotide deletion of nucleotide 111 (Δ111) (Figure 4.4). As U5 snRNA mutants are often lethal, due to the highly conserved

nature of the snRNA and the fact that U5 snRNA is essential for cell viability, U5 mutants were constructed in the ROK4 plasmid (referred to as U5 + ins). This pRS314 plasmid contains the U5 snRNA gene with a 20 nucleotide insert (U5 + ins) in stem 2, between positions U121 and C122 (Figure 4.5A). Constructing U5 snRNA mutants in the U5 + ins plasmid will enable wild-type and mutant U5 snRNAs to be present in the same cell, and be differentiated between via size difference.

U5 snRNA mutants constructed in the U5 + ins plasmid were tested for viability using a plasmid shuffle assay in a yeast strain in which the gene encoding wild-type U5 snRNA, *SNR7*, was deleted. As U5 snRNA is essential, the U5 snRNA deletion was complemented with wild-type *SNR7* present on a *URA3* plasmid. This strain was transformed with U5 snRNA mutants and colonies were transferred to 5FOA containing media, at which point the presence of the *URA3* plasmid containing wild-type U5 was selected against. Growth on 5FOA containing media results in the mutant U5 snRNA being the sole source of U5 snRNA, and after growth of yeast at 16°C, room temperature (RT), 30°C and 37°C, it can be determined if the U5 mutant is viable, lethal or temperature sensitive. The viability of U5 + ins was tested to confirm that the presence of the 20 nucleotide insert does not affect viability compared to wild-type U5 snRNA. A negative control of an empty pRS414 vector containing no U5 snRNA was also tested. U5 deletion cells containing U5 + ins show no growth defect when compared to U5 deletion strain containing the wild-type U5 snRNA gene in a pRS314 plasmid. This indicates that the 20 nucleotide insert in U5 + ins does not affect the function of U5 snRNA (Figure 4.5B).

The U5 snRNA mutants were tested for viability and temperature sensitivity, via plasmid shuffle assays, along with wild-type U5 in pR314 and U5 + ins positive controls, and a pRS414 negative control, at 16°C, RT, 30°C and 37°C (Figure 4.6B). Of the U5 snRNA

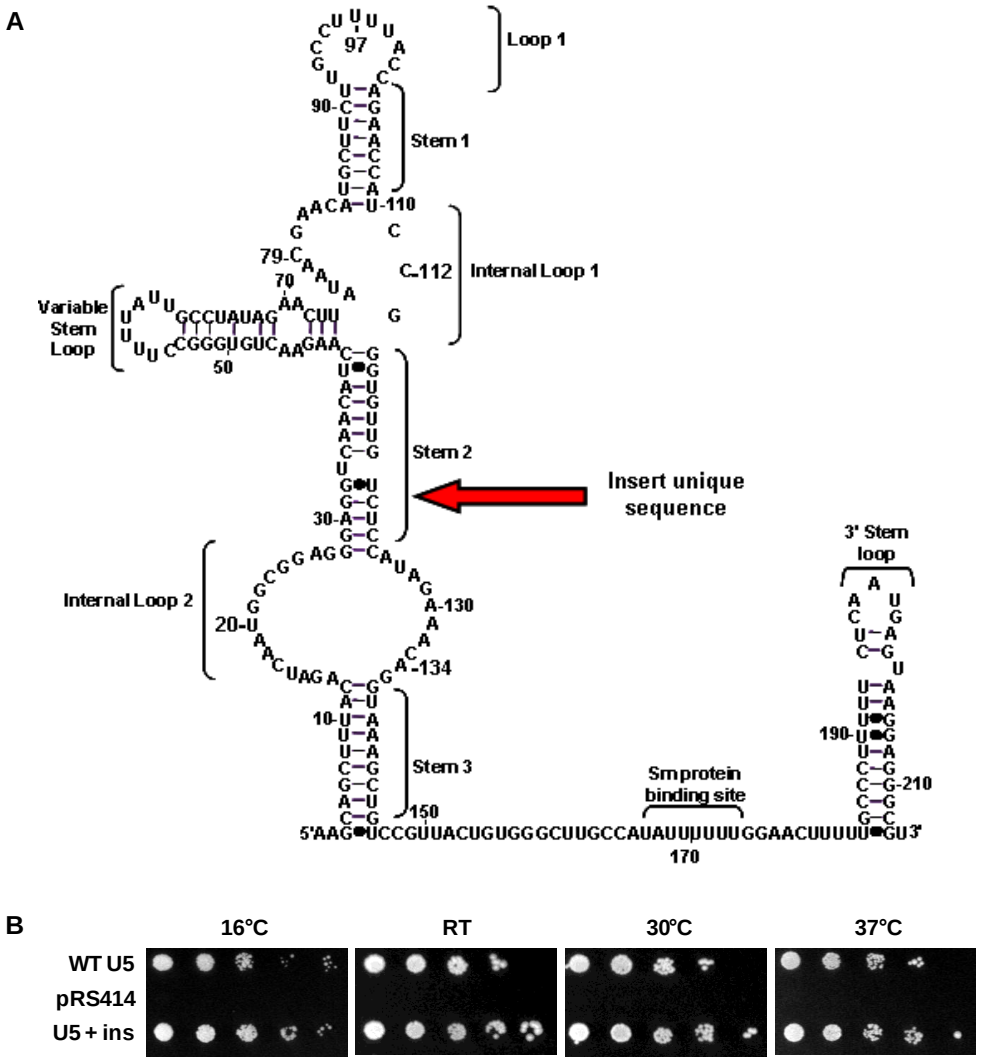
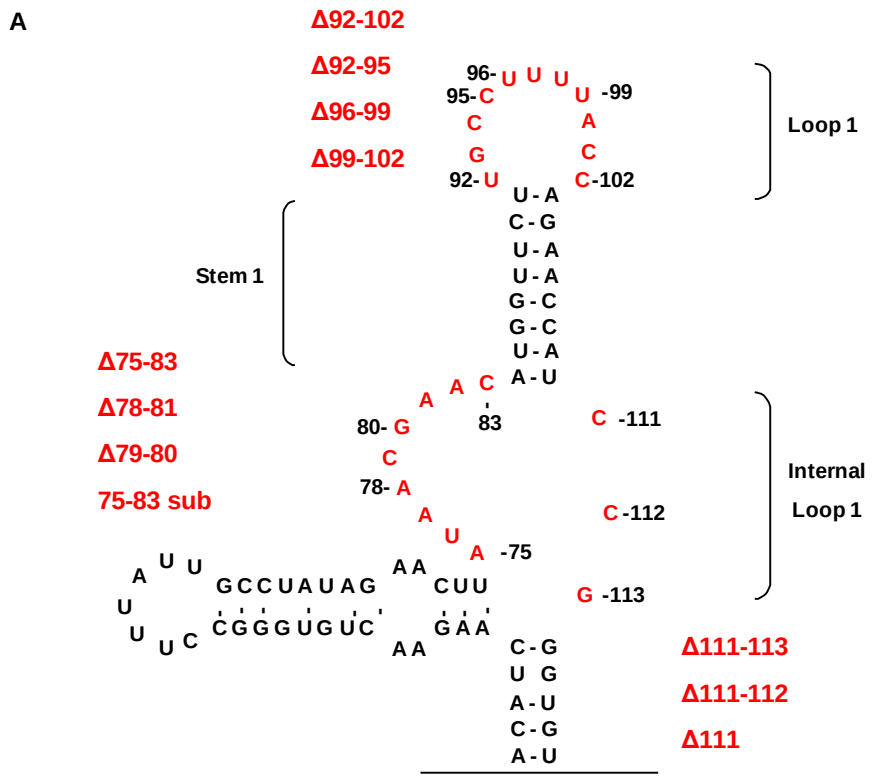


Figure 4.5 *In vivo* analysis of U5 + ins plasmid used for U5 snRNA mutant production. A. A 20 nucleotide sequence was inserted into stem 2 of U5 snRNA (U5 + ins) between nucleotides U121 and C122, allowing differentiation between mutant and wild-type U5 snRNAs due to difference in size. B. Plasmid shuffle assay to test if the 20 nucleotide insert in U5 snRNA (U5 + ins) effects viability of cells. A 1 in 5 serial dilution was performed starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. Each plate had a positive control strain containing wild-type U5 in pRS314, and a negative control strain containing pRS414. Spot plates were incubated at 16°C, room temperature (RT), 30°C and 37°C.


A
Δ92-102
Δ92-95
Δ96-99
Δ99-102
96-
95-C
C
G
92-U
U U U
U -99
A
C
C-102
Loop 1
Stem 1
U - A
C - G
U - A
U - A
G - C
G - C
U - A
A - U
Δ75-83
Δ78-81
Δ79-80
75-83 sub
A A C
83
80- G
C
78- A
A
U
A -75
C -111
C -112
G -113
Internal
Loop 1
A U U
G C C U A U A G
U
U C G G G U G U C
A A
C U U
G A A
C - G
U G
A - U
C - G
A - U
Δ111-113
Δ111-112
Δ111

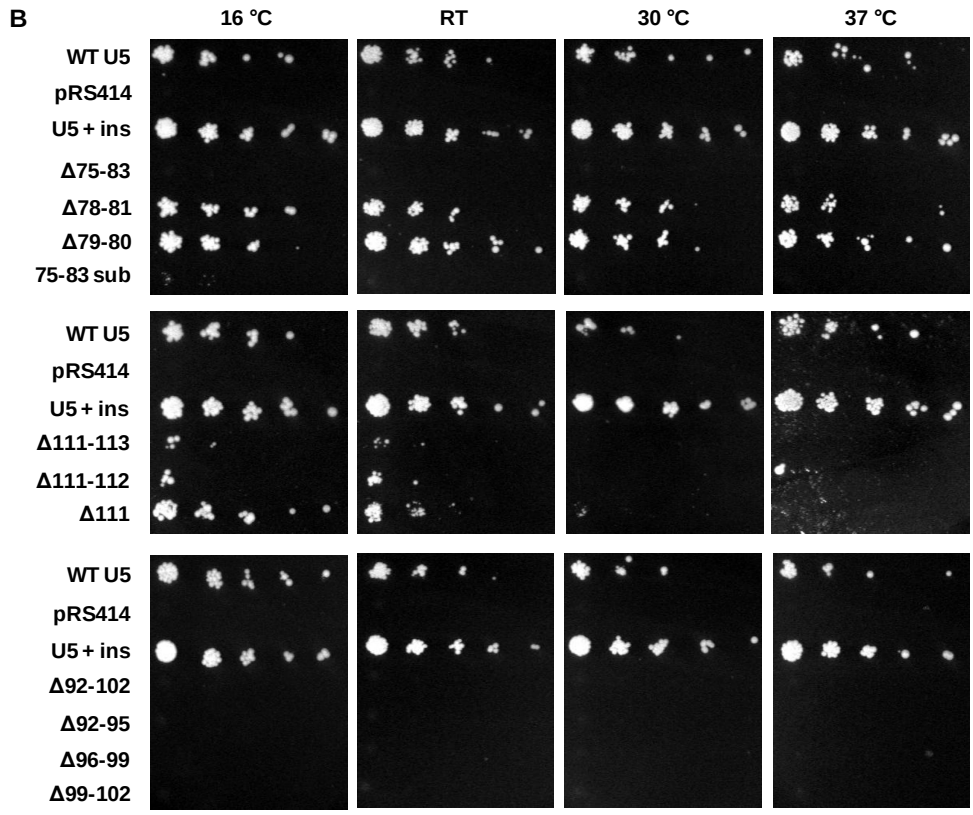

B
16 °C
RT
30 °C
37 °C
WT U5
pRS414
U5 + ins
Δ75-83
Δ78-81
Δ79-80
75-83 sub
WT U5
pRS414
U5 + ins
Δ111-113
Δ111-112
Δ111
WT U5
pRS414
U5 + ins
Δ92-102
Δ92-95
Δ96-99
Δ99-102

Figure 4.6 *In vivo* analysis of U5 snRNA mutants. A. Three regions of U5 snRNA were mutated to investigate the effects on cell viability. The three regions chosen for investigation were Loop 1 and the 3' and 5' side of Internal Loop 1 (IL1). These regions are highlighted in red on the diagram of the upper section of U5 snRNA. B. The three sets of U5 snRNA mutants were tested for viability via plasmid shuffle assays. A 1 in 5 serial dilution was carried out starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. Each plate had a positive control strain containing wild-type U5 on pRS414, and a negative control strain containing pRS314. Spot plates were incubated at 16°C, room temperature (RT), 30°C and 37°C.

mutations in the 3' side of IL1, Δ75-83 and 75-83 sub were both lethal and 78-81 del and 79-80 del were both viable, at 16°C, RT, 30°C and 37°C (Figure 4.6B). All four of the U5 Loop 1 mutants, Δ92-102, Δ92-95, Δ96-99 and Δ99-102, were lethal at 16°C, RT, 30°C and 37°C (Figure 4.6B). Δ111-113, Δ111-112 and Δ111 were lethal at 30°C and 37°C, and sick at RT and 16°C, showing reduced growth at RT and 16°C (Figure 4.6B).

4.1.2.2 Effects of U5 snRNA mutations on association of Brr2p, Snu114p and Prp8p with the U5 snRNA

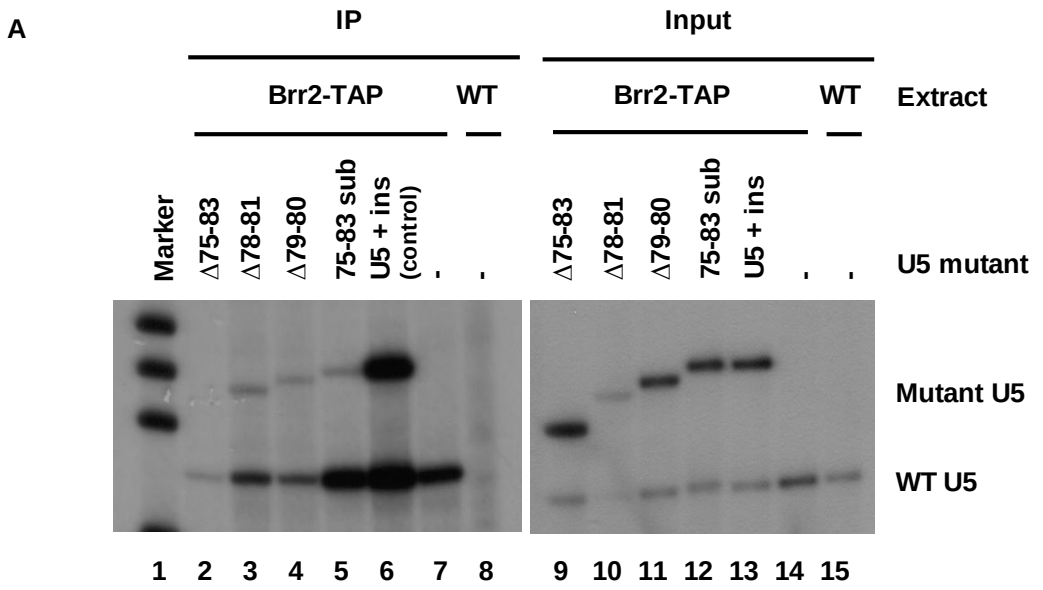
To investigate the effects of the U5 snRNA mutants on associations of Brr2p, Snu114p and Prp8p with U5 snRNA, and define the regions of U5 snRNA involved in these associations, immunoprecipitations were performed to see if specific mutations in the U5 snRNA caused a reduction in association of Brr2p, Snu114p and Prp8p with U5 snRNA.

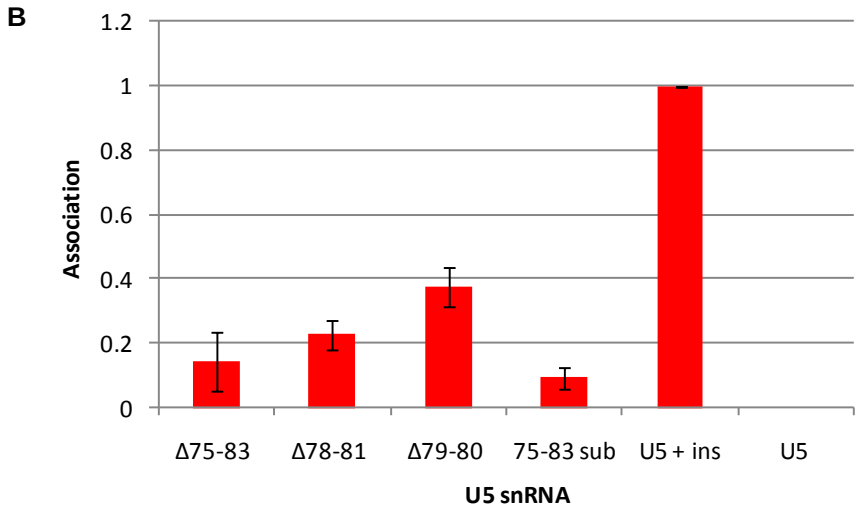
To investigate associations of Brr2p and Snu114p with U5 snRNA, whole cell extracts were produced from yeast containing TAP-tagged Brr2p or Snu114p, and both wild-type U5 snRNA (present in the genome) and mutant U5 snRNA (present on U5 + ins plasmid). Brr2p and Snu114p were immunoprecipitated via the TAP tag. To investigate associations of Prp8p with U5 snRNA, extracts were produced from yeast containing wild-type and mutant U5 snRNA, but with no tagged protein present. Prp8p was immunoprecipitated with an anti-Prp8p antibody. Following immunoprecipitation of Brr2p-TAP, Snu114p-TAP or Prp8p, associated RNA was purified and subjected to primer extension using a primer specific for U5 snRNA. Products from primer extensions were separated by denaturing PAGE and visualised by autoradiography (Figures 4.7 to 4.15A). Both associated wild-type and mutant U5 snRNAs were detected by the primer used. Mutant and wild-type U5 snRNA were differentiated by a size difference, with mutant U5 snRNA appearing larger due to the 20 nucleotide insert

present within U5 snRNA in the U5 + ins plasmid. A reduction in the amount of mutant U5 snRNA associated with the immunoprecipitated protein, compared to the level of associated U5 + ins without mutation, would suggest an involvement of the mutated region in associations with that protein. These experiments were repeated in triplicate and the amount of associated U5 snRNA was detected and quantified by phosphorimaging (Figures 4.7 to 4.15B). Primer extension was also carried out on total RNA from each extract to illustrate that both the wild-type and U5 snRNA mutants were present in each extract and could be detected by primer extension (Figures 4.7 to 4.15A). Western blotting was carried out on total protein from each extract, with an antibody to detect the protein to be immunoprecipitated and an antibody to detect Glucose-6-phosphate dehydrogenase (G6PD) as a loading control, to show that the presence of the U5 snRNA mutant did not affect levels of Brr2p, Snu114p and Prp8p in the extracts (Figures 4.7 to 4.15D).

4.1.2.3 Mutations in the 5' side of U5 snRNA Internal Loop 1 influence associations of Brr2p, Snu114p and Prp8p

To investigate how mutations in the 5' side of U5 snRNA IL1 effect associations of Brr2p, Snu114p and Prp8p with U5 snRNA, and to determine if nucleotides in this region are required for associations with Brr2p, Snu114p and Prp8p, immunoprecipitations were carried out using extracts containing mutant and wild-type U5 snRNAs (Figures 4.7 to 4.9A). Levels of U5 snRNA associated with Brr2p, Snu114p and Prp8p were detected by primer extension and phosphorimaging. All four mutations in the 5' side of Internal Loop 1 of U5 snRNA affected the association of U5 snRNA with Brr2p, Snu114p and Prp8p (Figures 4.7 to 4.9, B). In the case of Brr2p, the large deletion of 75-83 (Δ73-83) and the complimentary sequence (75-83 sub) had the largest effect, with the amount of U5 snRNA being immunoprecipitated


A
IP
Input
Brr2-TAP
WT
Brr2-TAP
WT
Extract
Marker
Δ75-83
Δ78-81
Δ79-80
75-83 sub
U5 + ins (control)
-
-
Δ75-83
Δ78-81
Δ79-80
75-83 sub
U5 + ins
-
-
U5 mutant
Mutant U5
WT U5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
1.2
1
0.8
0.6
0.4
0.2
0
Association
Δ75-83
Δ78-81
Δ79-80
75-83 sub
U5 + ins
U5
U5 snRNA

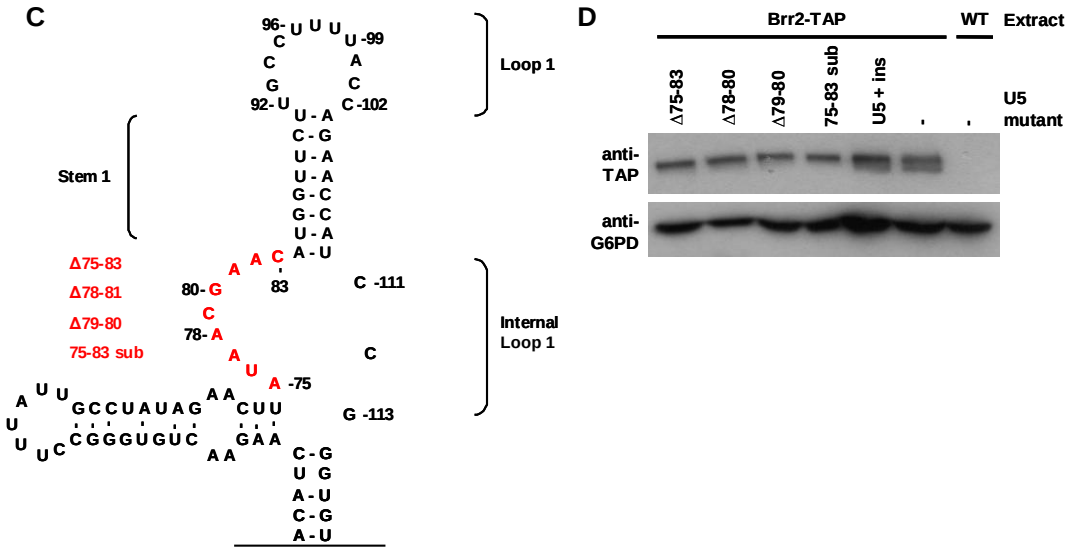
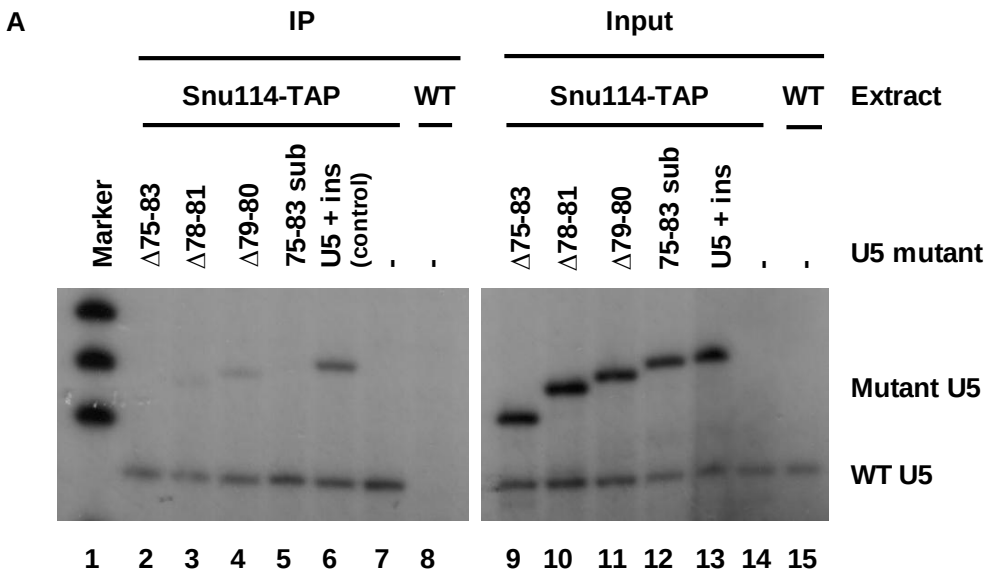

C
Loop 1
Stem 1
Internal Loop 1
Δ75-83
Δ78-81
Δ79-80
75-83 sub
D
Brr2-TAP
WT
Extract
Δ75-83
Δ78-80
Δ79-80
75-83 sub
U5 + ins
-
-
U5 mutant
anti-TAP
anti-G6PD

Figure 4.7 Effects of mutations in the 5' side of U5 snRNA Internal Loop 1 on Brr2p association. A. Immunoprecipitation (IP) of Brr2-TAP was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 8). Negative control using untagged extract was performed (lane 8). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 9 to 15). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lane 1 contains 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Brr2p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated in triplicate and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, the 5' side of U5 snRNA IL1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Brr2p. Brr2p levels were detected using anti-TAP antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.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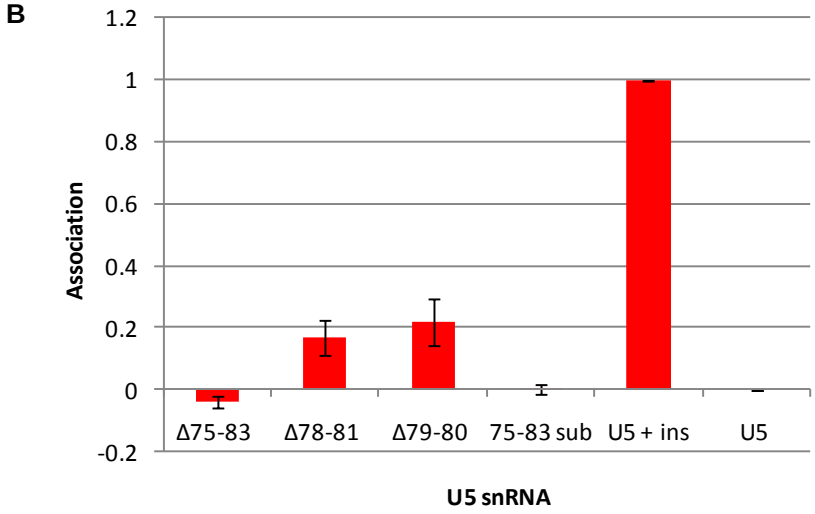
IP
Input
Snu114-TAP
WT
Snu114-TAP
WT
Extract
Marker
Δ75-83
Δ78-81
Δ79-80
75-83 sub
U5 + ins (control)
-
-
Δ75-83
Δ78-81
Δ79-80
75-83 sub
U5 + ins
-
-
U5 mutant
Mutant U5
WT U5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15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0.6
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Association
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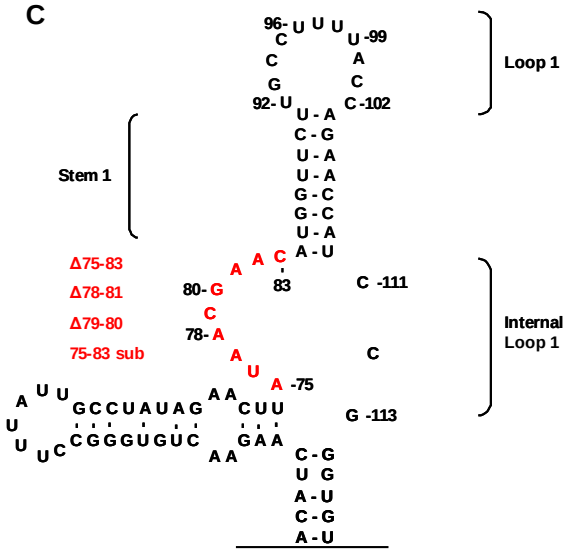
Δ78-81
Δ79-80
75-83 sub
U5 + ins
U5
U5 snRNA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Stem 1
Internal Loop 1
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Δ78-81
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75-83 sub
96-
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92-
-102
80-
83
78-
-111
-75
-113

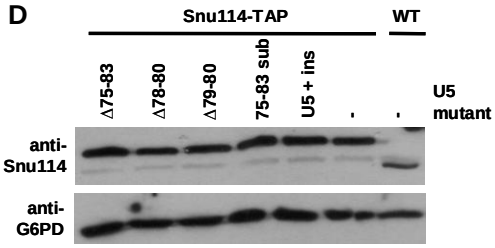
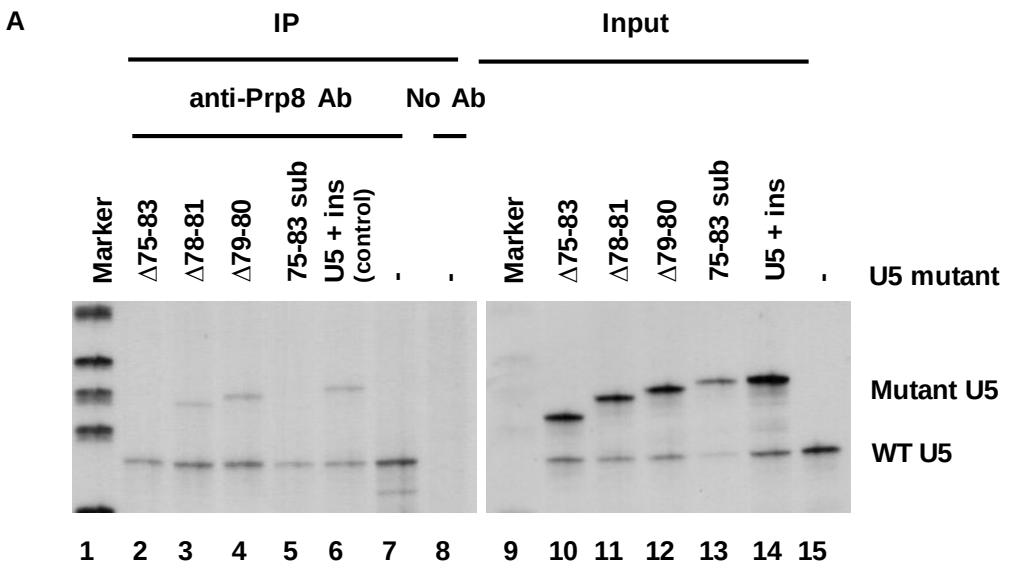

D
Snu114-TAP
WT
Δ75-83
Δ78-80
Δ79-80
75-83 sub
U5 + ins
-
-
U5 mutant
anti-Snu114
anti-G6PD

Figure 4.8 Effects of mutations in the 5' side of U5 snRNA Internal Loop 1 on Snu114p association. A. Immunoprecipitation (IP) of Snu114-TAP was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 8). Negative control using untagged extract was performed (lane 8). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 9 to 15). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lane 1 contains 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Snu114p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated in triplicate and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, the 5' side of U5 snRNA IL1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Snu114p. Snu114p levels were detected using anti-Snu114 antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.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anti-Prp8 Ab
No Ab
Marker
Δ75-83
Δ78-81
Δ79-80
75-83 sub
U5 + ins (control)
-
-
Marker
Δ75-83
Δ78-81
Δ79-80
75-83 sub
U5 + ins
-
U5 mutant
Mutant U5
WT U5
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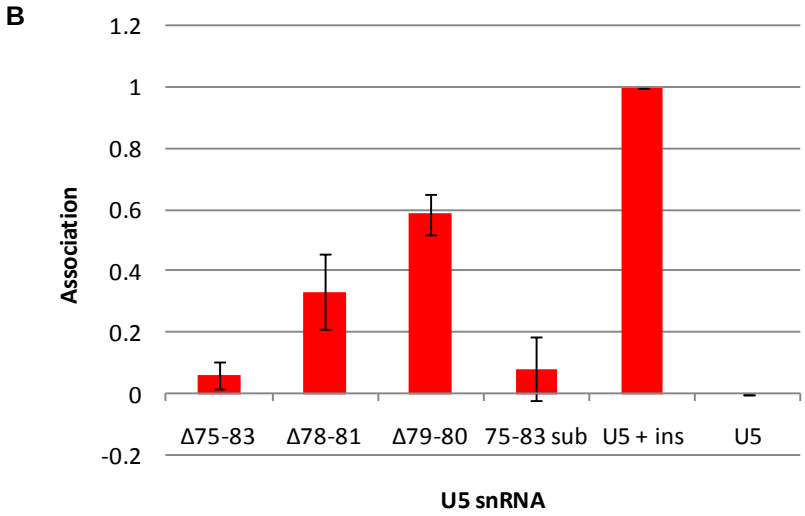
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0.8
0.6
0.4
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Association
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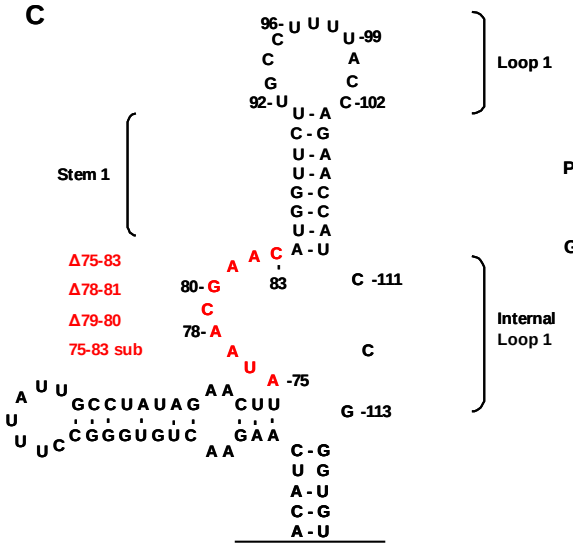
Δ78-81
Δ79-80
75-83 sub
U5 + ins
U5
U5 snRNA


C
Loop 1
Stem 1
Internal Loop 1
Δ75-83
Δ78-81
Δ79-80
75-83 sub

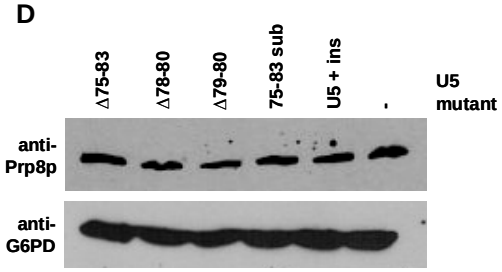

D
Δ75-83
Δ78-80
Δ79-80
75-83 sub
U5 + ins
-
U5 mutant
anti-Prp8p
anti-G6PD

Figure 4.9 Effects of mutations in the 5' side of U5 snRNA Internal Loop 1 on Prp8p association. A. Immunoprecipitation (IP) of Prp8p was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 8). Negative control using no Prp8p antibody was performed (lane 8). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 10 to 15). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lanes 1 and 9 contain 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Prp8p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated in triplicate and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, the 5' side of U5 snRNA IL1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Prp8p. Prp8p levels were detected using anti-Prp8 antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.

being reduced by 86 and 91%, respectively, compared to the U5 + ins without mutation (Figure 4.7B). The smaller deletions, Δ78-81 and Δ79-80, also affected associations of Brr2p with U5 snRNA. The levels of mutant U5 snRNA being immunoprecipitated with Δ78-81 and Δ79-80 were reduced by 77 and 62% respectively, compared to U5 + ins without mutation (Figure 4.7B).

Similar to that found with Brr2p, the 75-83 deletion mutation (Δ75-83) and the sequence substitution (75-83 sub) had the largest effect on Snu114p association with U5 snRNA, with almost none of either of these mutant U5 snRNAs being associated with Snu114p (Figure 4.8B). The amount of Δ78-81 and Δ79-80 U5 snRNA associated with Snu114p were reduced to just 17 and 22% compared to U5 + ins without mutation (Figure 4.8B).

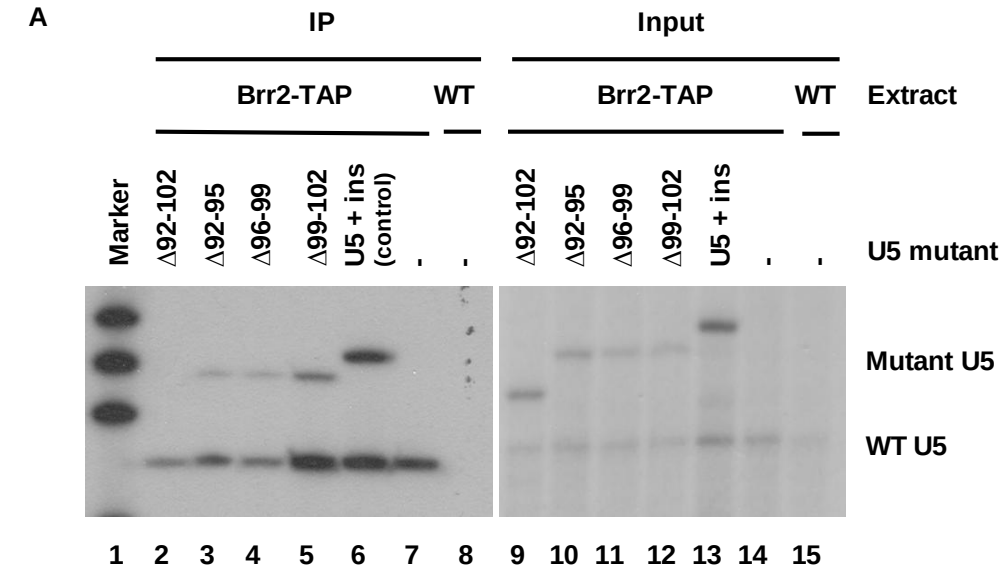
Continuing the trend seen with Brr2p and Snu14p, the largest effect on associations between Prp8p and U5 snRNA was seen with Δ75-83 and 75-83 sub U5 snRNAs. In both cases the amount of mutant U5 snRNA associated with Prp8p was reduced by 90% compared to U5 + ins without mutation (Figure 4.9B). Deletion of nucleotides 78 to 81 of U5 snRNA (Δ78-81) displayed a reduction in association with Prp8p, by 67% compared to U5 + ins without mutation (Figure 4.9B). However, the effect of deleting nucleotides 79 to 80 of U5 snRNA (Δ79-80) were not as drastic, with U5 snRNA association only reduced by 41% compared to U5 + ins without mutation (Figure 4.9B).

In all cases, the presence of the U5 snRNA 5' IL1 mutants did not affect the levels of Brr2p, Snu114p or Prp8p (Figures 4.7 to 4.9D). The general trend observed in these experiments is that deletion of nucleotides 75 to 83 (Δ75-83) of U5 snRNA almost abolishes association of Brr2p, Snu114p and Prp8p with U5 snRNA. Although deletion of nucleotides 78 to 81 (Δ78-81) and 79 to 80 (Δ79-80) of U5 snRNA do effect associations of Brr2p,

Snu114p and Prp8p, the effect is not as drastic as with Δ75-83. Brr2p, Snu114p and Prp8p do still associate to some extent with Δ78-81 and Δ79-80 U5 snRNA mutants. This would indicate that larger deletions of IL1 have a larger effect of protein association with U5 snRNA, and that size of the 5' side of U5 snRNA IL1 is important. However, substituting nucleotides 75 to 83 (75-83 sub) with the reverse complement of the wild-type sequence also almost abolished the association of Brr2p, Snu114p and Prp8p. This indicates that the sequence of the 5' side of U5 snRNA IL1, not just size, is important for association of Brr2p, Snu114p and Prp8p with U5 snRNA. The association of Snu114p with U5 snRNA appears to be most sensitive to mutations in the 5' side of U5 snRNA IL1, and association of Prp8p with U5 snRNA is most resilient to mutations in this region.

4.1.2.4 Mutations in U5 snRNA Loop 1 influences associations of Brr2p, Snu114p and Prp8p

Immunoprecipitation and primer extension was carried out, from extracts containing wild-type and mutant U5 snRNA, to investigate how deletions in U5 snRNA Loop 1 effect association of Brr2p, Snu114p and Prp8p with U5 snRNA. All of the U5 snRNA Loop 1 mutations affect association of Brr2p. The largest deletion, Δ92-102 U5 snRNA, had the most drastic effect on Brr2p associations with mutant U5 snRNA, with levels of associated Δ92-102 U5 snRNA being reduced by 91% compared to U5 + ins without mutation (Figure 4.10B). Of the three smaller deletions in Loop 1 of four nucleotides each, Δ92-95 and Δ99-102 had the largest effect, with levels of associated mutant U5 snRNA being reduced by 52 and 62% respectively, compared to U5 + ins without mutation (Figure 4.10B). Deletion of nucleotides 96 to 99 (Δ96-99) of U5 snRNA has slightly less effect than the other two small deletions, with amounts of associated mutant U5 snRNA being reduced by 41% compared to U5 + ins without mutation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Δ99-102
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-
Δ92-102
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U5 mutant
Mutant U5
WT U5
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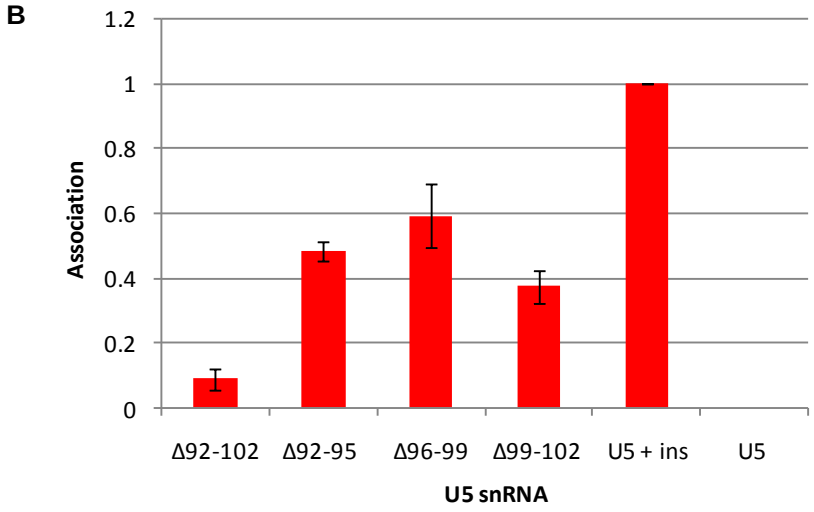
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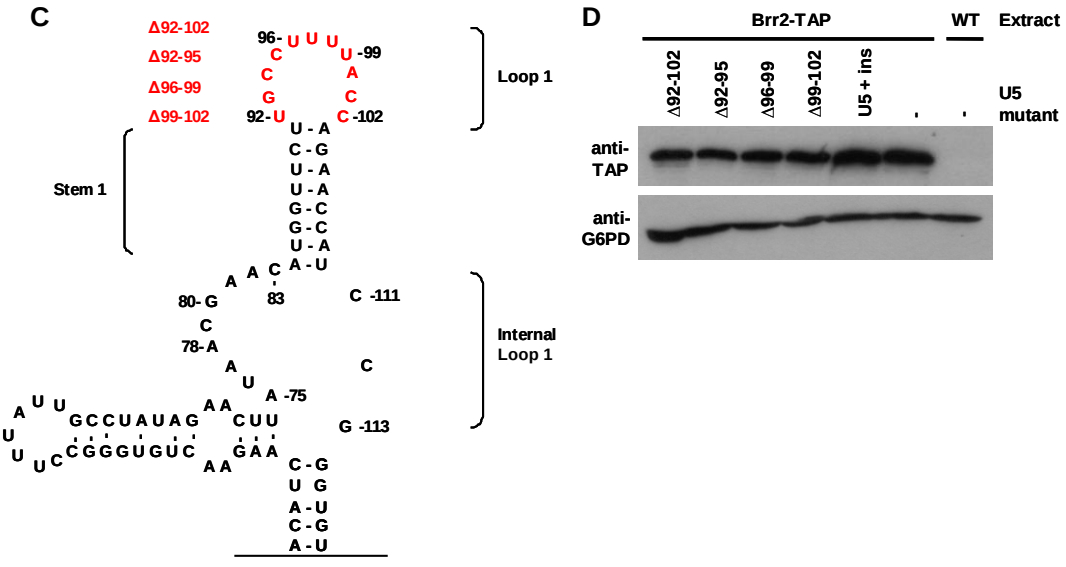
Δ99-102
U5 + ins
U5
U5 snRNA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Δ92-95
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Δ99-102
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Stem 1
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Extract
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Δ92-95
Δ96-99
Δ99-102
U5 + ins
-
-
U5 mutant
anti-TAP
anti-G6PD

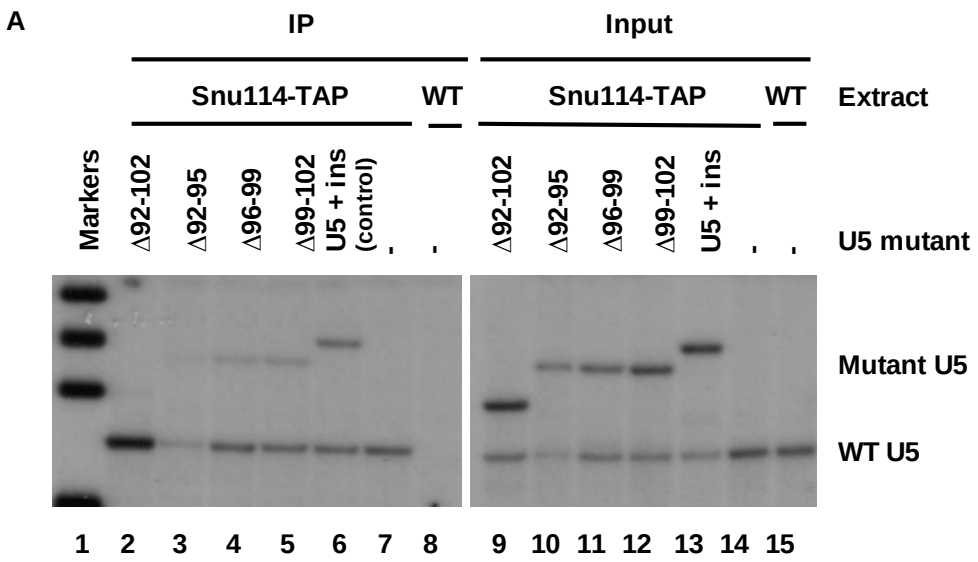
Figure 4.10 Effects of mutations in U5 snRNA Loop 1 on Brr2p association. A. Immunoprecipitation (IP) of Brr2-TAP was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 8). Negative control using untagged extract was performed (lane 8). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 9 to 15). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lane 1 contains 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Brr2p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated in triplicate and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, U5 snRNA Loop 1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Brr2p. Brr2p levels were detected using anti-TAP antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.

(Figure 4.10B). These results indicate that of the nucleotides present in U5 Loop 1, nucleotides 99 to 102 are most important for the association of Brr2p with U5 snRNA.

All of the deletions made in U5 snRNA Loop 1 also effect associations of Snu114p with the U5 snRNA. The effects of deleting U5 snRNA Loop 1 nucleotides 92 to 102 (Δ92-102) did not have as large an effect as with Brr2p, as association of mutant U5 snRNA was reduced by 72% compared to U5 + ins without mutation (Figure 4.11B). The three smaller U5 snRNA Loop 1 deletions, Δ92-95, Δ96-99 and Δ99-102, reduced association of Snu114p with mutant U5 snRNA by 62, 42 and 26%, respectively, compared to U5 + ins without mutation (Figure 4.11B). This suggests that nucleotides 92 to 95 are more important than nucleotides 96 to 102 for the association of Snu114p with U5 snRNA.

The largest U5 snRNA Loop 1 deletion, Δ92-102, reduced Prp8p association with mutant U5 snRNA by 89% compared to U5 + ins without mutation (Figure 4.12B). All of the smaller deletions also affected association of Prp8p with mutant U5 snRNA. Each of the smaller deletions (Δ92-95, Δ96-99 and Δ92-102) had similar effects on Prp8p associations with U5 snRNA, reducing associations of Prp8p with mutant U5 snRNA by 50 and 45% compared to U5 + ins without mutation (Figure 4.12B). The fact the three smaller deletions all had similar effects may suggest that the size of U5 snRNA Loop 1, not just sequence, is important for Prp8p association with U5 snRNA.

The presence of the U5 snRNA Loop 1 mutants did not affect the levels of Brr2p, Snu114p and Prp8p (Figures 4.10 to 4.12D). Of the U5 snRNA Loop 1 mutants tested, deletion of nucleotides 92-102 of U5 snRNA Loop 1 (Δ92-102) has the largest effect on association of Brr2p, Snu114p and Prp8p. The three smaller deletions (Δ92-95, Δ96-99 and Δ99-102) also effected associations of Brr2p, Snu114p and Prp8p with U5 snRNA, but associations of Brr2p, Snu114p and Prp8 with U5 snRNA are not affected in the same way by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-
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WT U5
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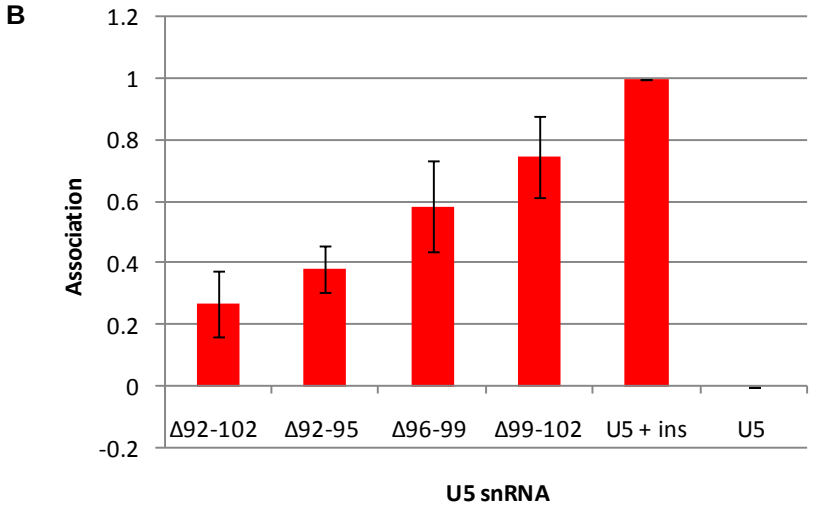
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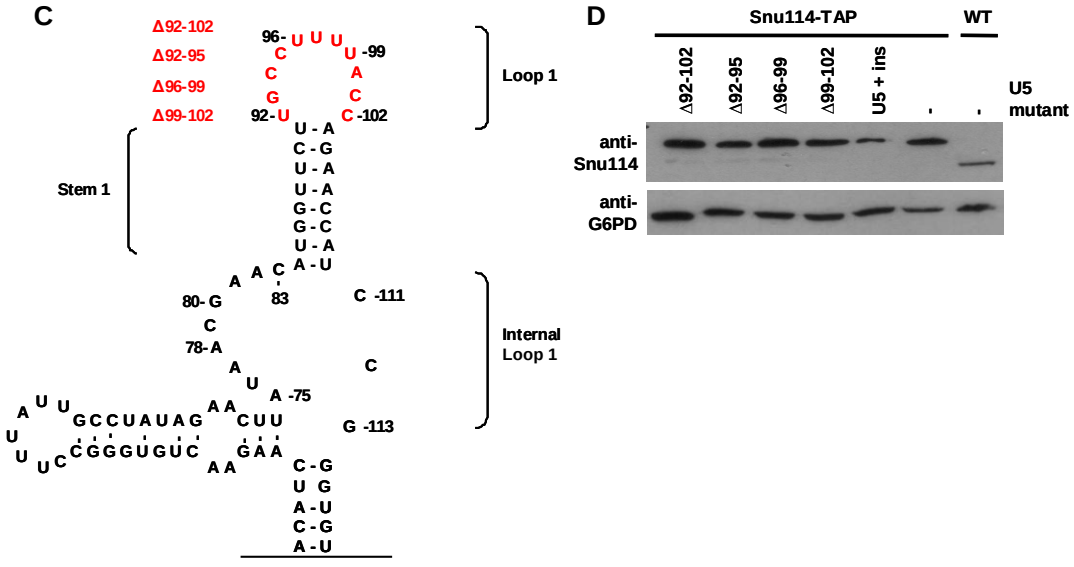
U5 snRNA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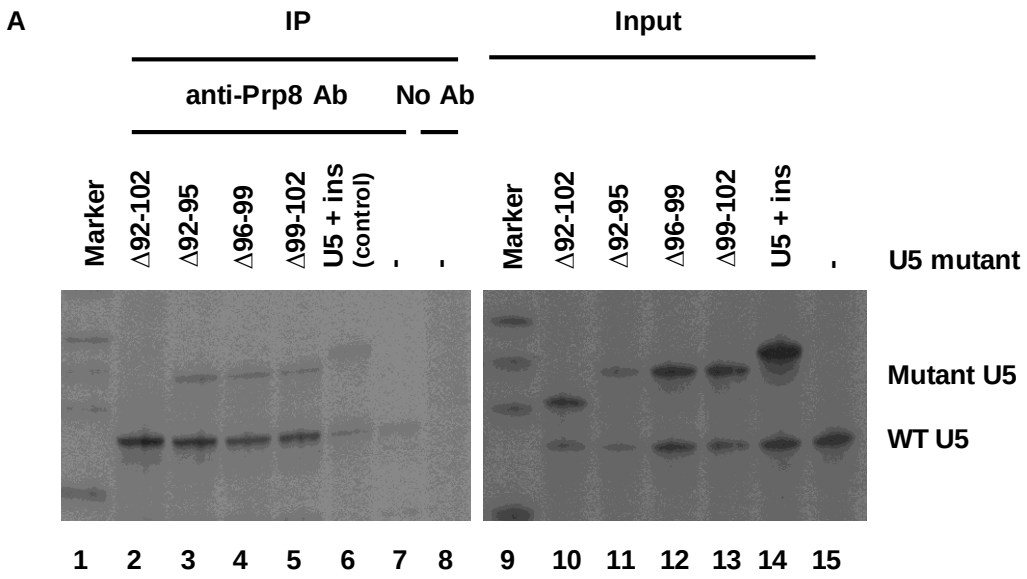
Δ96-99
Δ99-102
U5 + ins
-
-
U5 mutant
anti-Snu114
anti-G6PD

Figure 4.11 Effects of mutations in U5 snRNA Loop 1 on Snu114p association. A. Immunoprecipitation (IP) of Snu114-TAP was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 8). Negative control using untagged extract was performed (lane 8). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 9 to 15). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lane 1 contains 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Snu114p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated in triplicate and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, U5 snRNA Loop 1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Snu114p. Snu114p levels were detected using anti-Snu114 antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.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Δ96-99
Δ99-102
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-
Marker
Δ92-102
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-
U5 mutant
Mutant U5
WT U5
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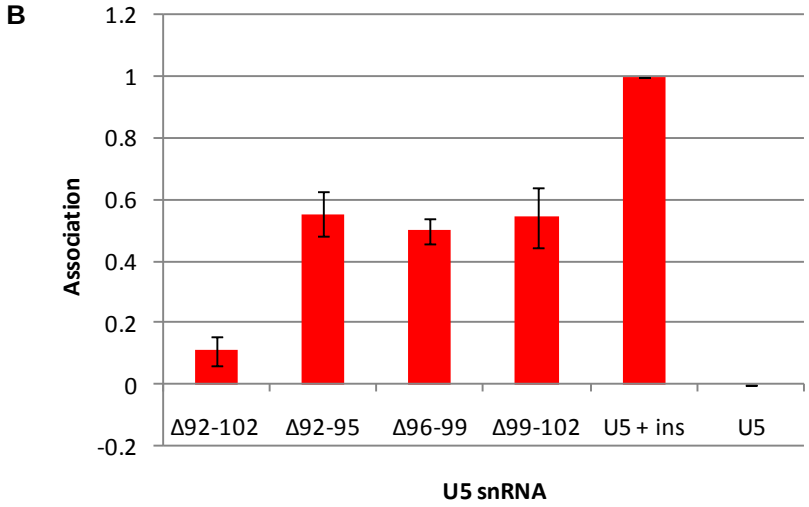
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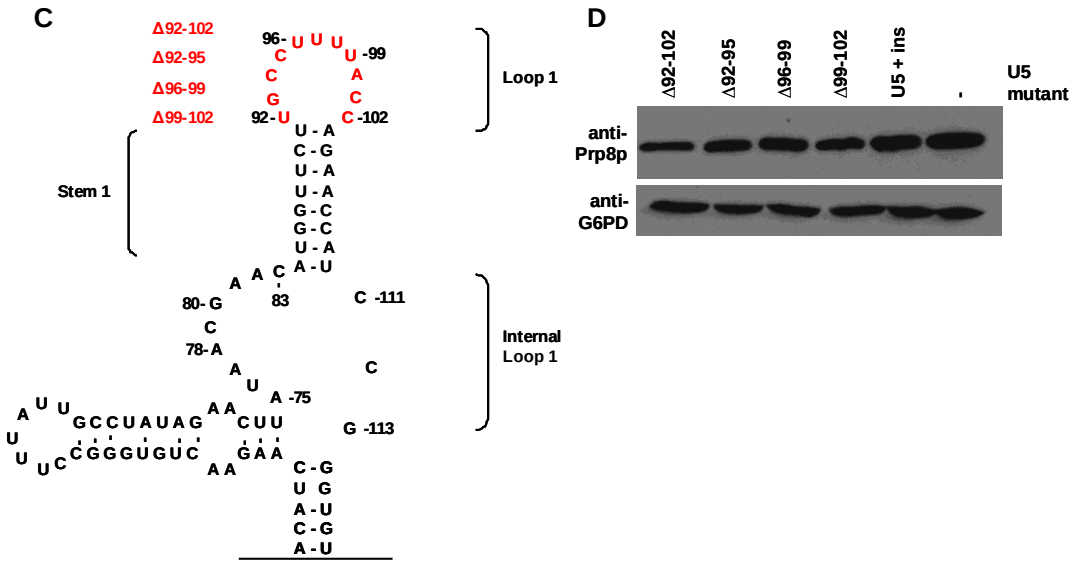
U5 snRNA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Δ99-102
U5 + ins
-
U5 mutant
anti-Prp8p
anti-G6PD

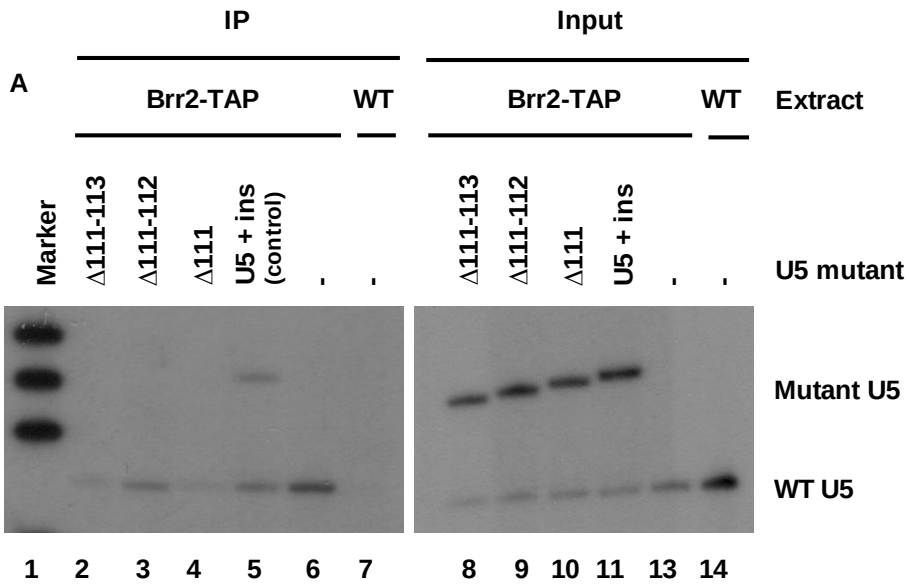
Figure 4.12 Effects of mutations in U5 snRNA Loop 1 on Prp8p association. A. Immunoprecipitation (IP) of Prp8p was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 8). Negative control using no Prp8p antibody was performed (lane 8). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 10 to 15). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lanes 1 and 9 contain 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Prp8p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated in triplicate and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, U5 snRNA Loop 1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Prp8p. Prp8p levels were detected using anti-Prp8 antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.

each of these mutations. Deletion of nucleotides 92 to 95 of U5 snRNA Loop 1 (Δ92-95) had the largest effect of associations of Snu114p and deletion of U5 snRNA Loop 1 nucleotides 99 to 102 (Δ92-102) had the largest effect of association of Brr2p with U5 snRNA. Each of the smaller deletions had a very similar effect on the association of Prp8p with U5 snRNA, indicating that the size of U5 snRNA loop 1 is important for associations between U5 snRNA and Prp8p.

4.1.2.5 Mutations in the 3' side of U5 snRNA internal loop 1 influence associations of Brr2p, Snu114p and Prp8p

Immunoprecipitations from extracts containing wild-type and mutant U5 snRNA were performed, followed by primer extension, to investigate the effect of deletion in the 3' side of U5 snRNA IL1 on association of Brr2p, Snu114p and Prp8p with U5 snRNA. These experiments will also determine if the 3' side of U5 snRNA IL1 is required for association of Brr2p, Snu114p and Pp8p with the U5 snRNA. All deletions in the 3' side of U5 snRNA IL1 reduce association of U5 snRNA with Brr2p by 89% or more, and Snu114p by more than 94%, compared to U5 + ins without mutation (Figure 4.13B and 4.14B). In the case of Prp8p, deletion of nucleotides 111 to 113 (Δ111-113) and 111 to 112 (Δ111-112) of U5 snRNA reduced associations by 95 and 93%, respectively, compared to U5 + ins without mutation (Figure 4.15B). Deletion of U5 snRNA nucleotide 111 (Δ111) reduced association of mutant U5 snRNA with Prp8p by 83% compared to U5 + ins without mutation (Figure 4.15B).

Western blotting revealed that the presence of U5 snRNA containing deletions in the 3' side of IL1 does not affect levels of Brr2p, Snu114p or Prp8p (Figure 4.12 to 4.15D). These results demonstrate that association of Brr2p, Snu114p and Prp8p with the U5 snRNA is very sensitive to deletions in the 3' side of U5 snRNA IL1.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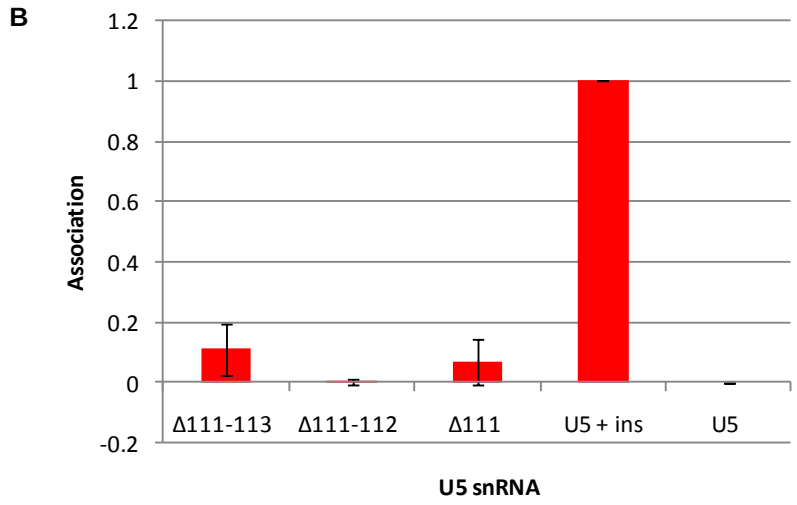
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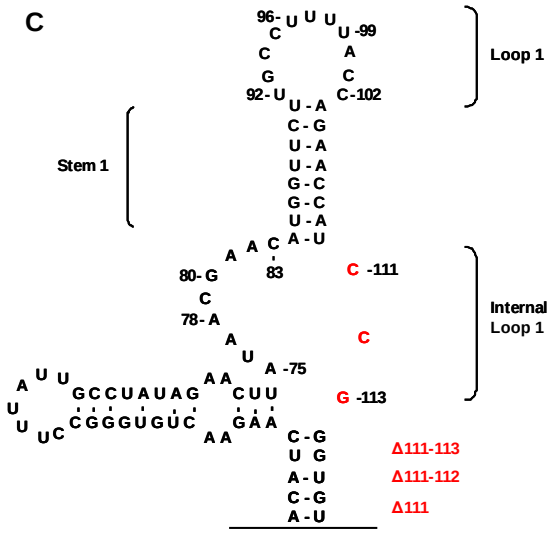
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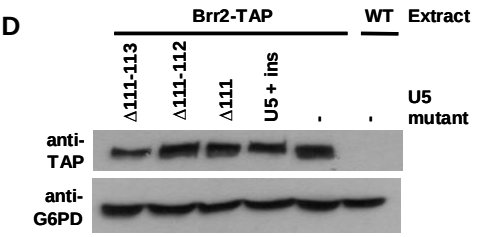
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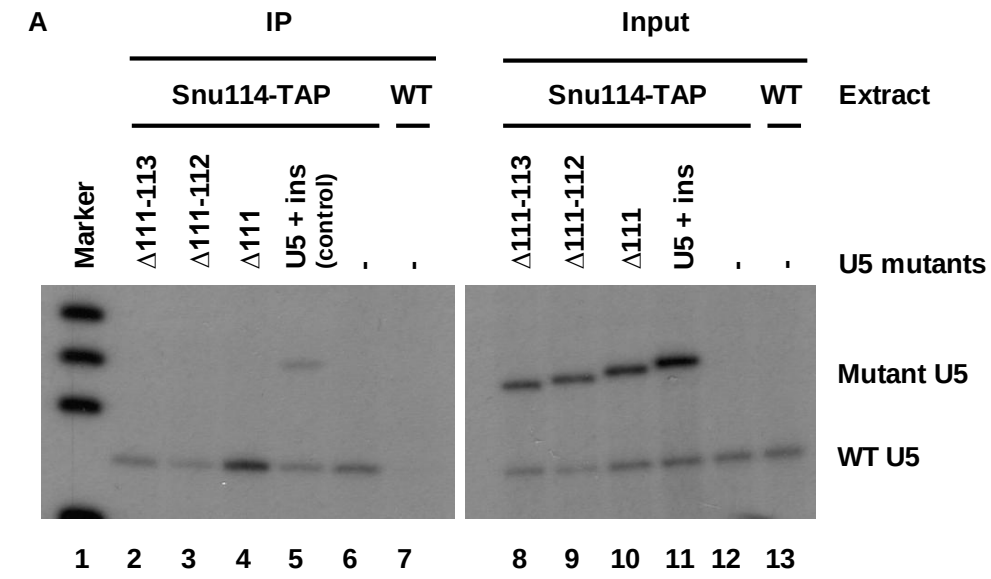
-
-
U5 mutant
anti-TAP
anti-G6PD

Figure 4.13 Effects of mutations in the 3' side of U5 snRNA Internal Loop 1 on Brr2p association. A. Immunoprecipitation (IP) of Brr2-TAP was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 7). Negative control using untagged extract was performed (lane 7). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 8 to 14). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lane 1 contains 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Brr2p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated in triplicate and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, the 3' side of U5 snRNA IL1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Brr2p. Brr2p levels were detected using anti-TAP antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.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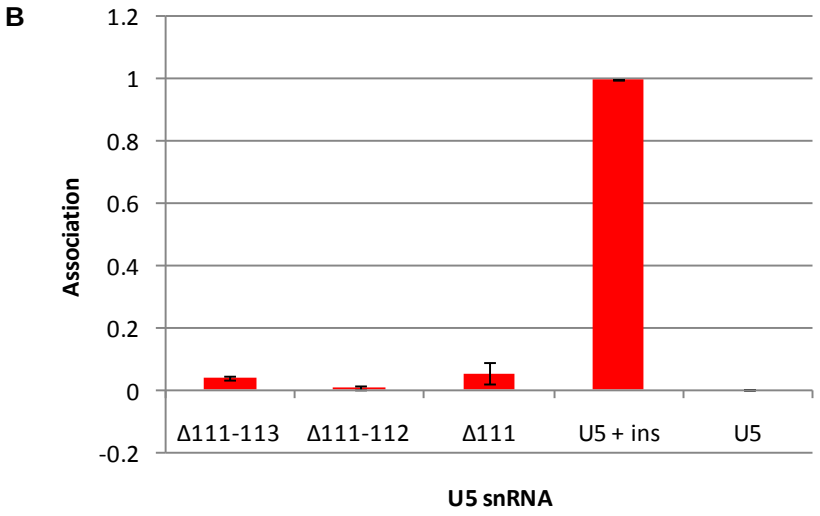
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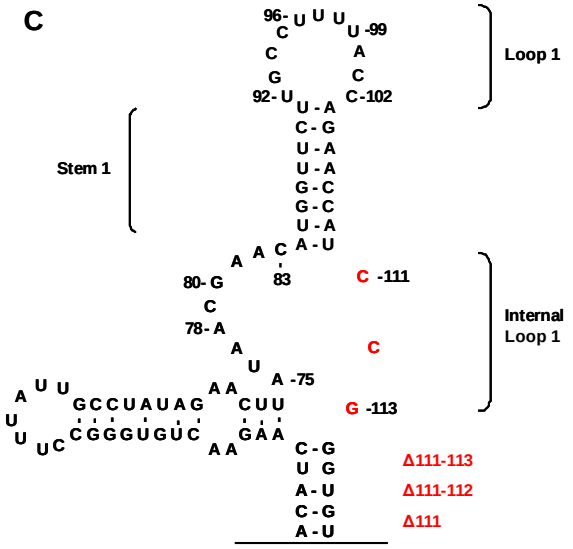
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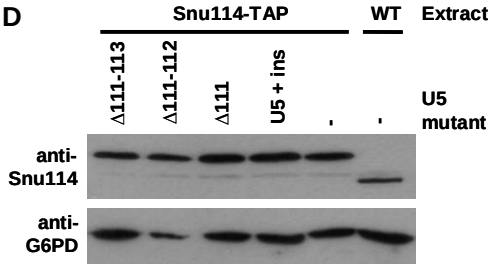
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Extract
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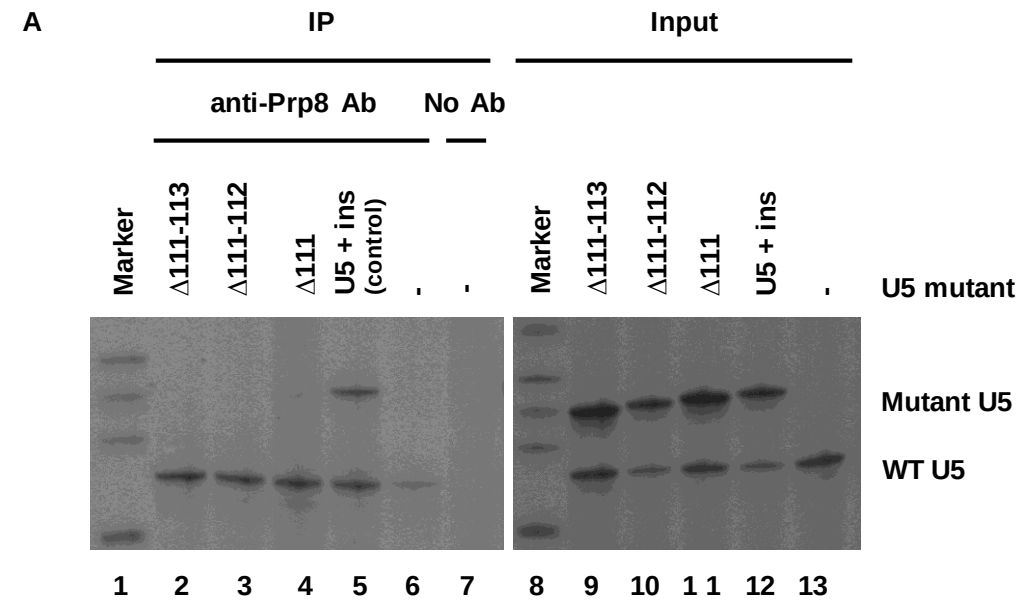
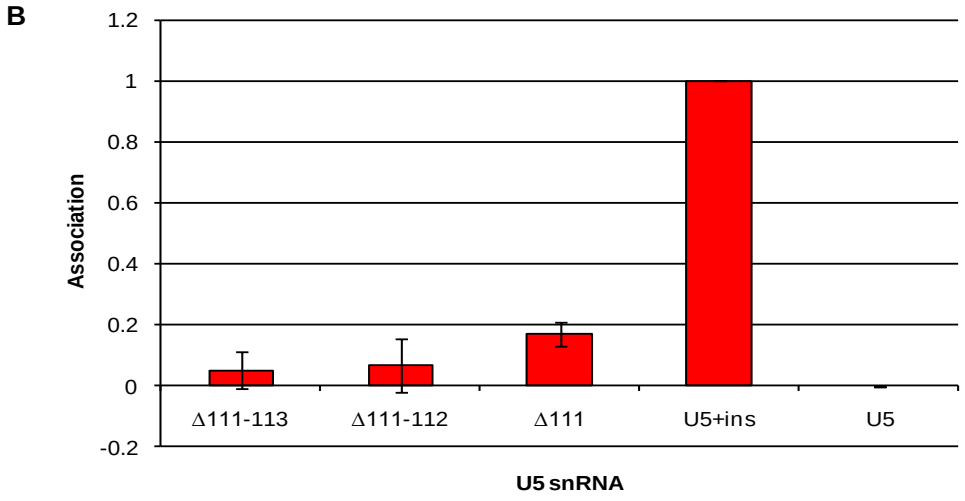
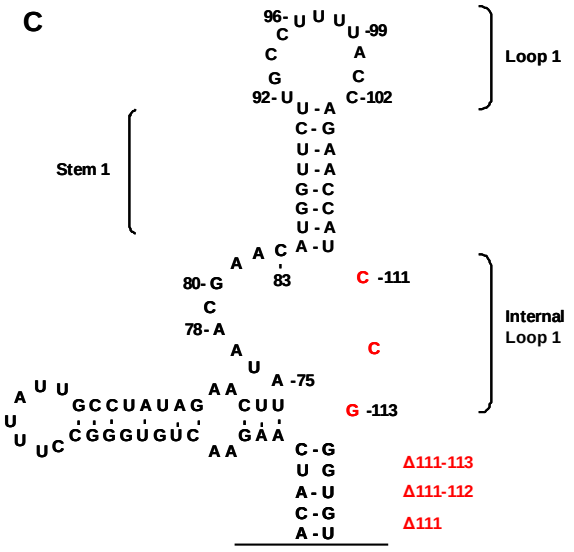
-
-
U5 mutant
anti-Snu114
anti-G6PD

Figure 4.14 Effects of mutations in the 3' side of U5 snRNA Internal Loop 1 on Snu114p association. A. Immunoprecipitation (IP) of Snu114-TAP was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 7). Negative control using untagged extract was performed (lane 7). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 8 to 14). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lane 1 contains 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Snu114p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated twicw and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, the 3' side of U5 snRNA IL1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Snu114p. Snu114p levels were detected using anti-Snu114 antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.


A
IP
Input
anti-Prp8 Ab
No Ab
Marker
Δ111-113
Δ111-112
Δ111
U5 + ins (control)
-
-
Marker
Δ111-113
Δ111-112
Δ111
U5 + ins
-
U5 mutant
Mutant U5
WT U5
1 2 3 4 5 6 7 8 9 10 1 1 12 13


B
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Association
Δ111-113
Δ111-112
Δ111
U5+ins
U5
U5 snRNA


C
Loop 1
Stem 1
Internal Loop 1
Δ111-113
Δ111-112
Δ111

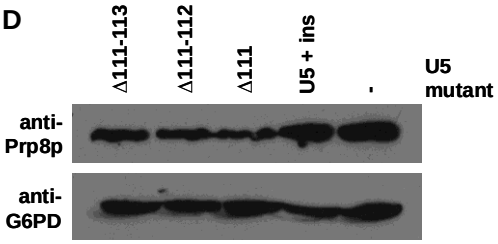

D
Δ111-113
Δ111-112
Δ111
U5 + ins
-
U5 mutant
anti-Prp8p
anti-G6PD

Figure 4.15 Effects of mutations in the 3' side of U5 snRNA Internal Loop 1 on Prp8p association. A. Immunoprecipitation (IP) of Prp8p was carried out from extracts containing wild-type and mutant U5 snRNA. RNA associated with the immunoprecipitated protein was isolated and subjected to primer extension using a primer specific to U5 snRNA (lanes 2 to 7). Negative control using no Prp8p antibody was performed (lane 7). Total RNA from each extract was also subjected to primer extension using primer specific to U5 snRNA (input, lanes 9 to 13). U5 snRNA mutants were constructed in a plasmid containing U5 snRNA with a 20 nucleotide insert (U5 + ins). Therefore, U5 snRNA mutants (Mutant U5) are detected as a larger product than wild-type U5 snRNA (WT U5). Lanes 1 and 8 contain 32P labelled pBR322 MspI-digested DNA. B. Graphical illustration of the amount of mutant U5 snRNA associated with Prp8p in comparison to levels of associated U5 + ins without mutation. The experiment shown in panel A was repeated in triplicate and quantitated by phosphorimaging. Error bars on each column represent the standard error. C. The region of U5 snRNA mutated for this set of experiments, the 3' side of U5 snRNA IL1, is highlighted in red in this diagram of the upper section of U5 snRNA. D. Western blotting was carried out on total protein from each extract to prove that the presence of U5 snRNA mutants does not effect levels of Prp8p. Prp8p levels were detected using anti-Prp8 antibody and glucose-6-phosphate dehydrogenase (G6PD) was detected as a loading control using anti-G6PD antibody.

4.1.3 Synthetic lethal screen reveals genetic interactions between *BRR2* and 3' side U5 snRNA IL1

Although it is common knowledge that Brr2p is a major component of the U5 snRNP, little is known about the physical and genetic interactions of Brr2p with the U5 snRNA, and the regions of Brr2p involved in these interactions. To investigate genetic interactions between Brr2p and U5 snRNA, seven published and four novel *brr2* mutants were constructed (Figures 4.16, 4.17A, Table 4.1). The *brr2* mutants will be used in a synthetic lethal screen, along with U5 snRNA mutants, to detect any genetic interaction between the mutated regions of *BRR2* and U5 snRNA.

All of the *brr2* mutants constructed were tested for viability via plasmid shuffle using a *BRR2*/*SNR7* (U5 snRNA) deletion strain, which contained wild-type *BRR2* and U5 snRNA on a *URA3* plasmid. The *BRR2*/U5 snRNA deletion strain was co-transformed with a *brr2* mutant and wild-type U5 snRNA. Transformants were transferred to 5FOA containing media and tested for viability at 16°C, RT, 30°C and 37°C (Figure 4.17B). The novel *brr2* mutants containing mutations in the first helicase-like domain (H1), Brr2-P841L and Brr2-G873L, were both lethal at all temperatures tested (Figure 4.17B). The novel *brr2* N-terminal mutant, Brr2-R295I, and two *brr2* alleles containing mutations in the first Sec63 domain (S1), Brr2-N1104L and Brr2-F1149I, were viable at all temperatures tested (Figure 4.17B). Both *brr2* mutants containing mutations in the second helicase-like domain (H2), G1375D,K1376N and D1474G, were also viable at all temperatures tested (Figure 4.17B). The H1 mutant, Brr2-E610G was lethal at 16°C and viable at all other temperatures tested (Figure 4.17B). Brr2-E909K, containing a mutation in H1, was viable at 16°C, RT and 30°C, but lethal at 37° (Figure 4.17B). The S1 mutants, Brr2-R1107A and R1107L, are both lethal at 16 and 37°C, and viable at RT and 30°C (Figure 4.17B). The lethal phenotype of the two novel H1 mutants,

Table 4.1 Eleven *brr2* mutants were constructed for used in a synthetic lethal screen with U5 snRNA mutants

brr2 mutant	Reference	Description
R295I	This study	Arginine to isoleucine substitution in the N-terminal domain (NTD).
E610G	Raghunathan and Guthrie, 1998	Glutamic acid to glycine substitution in the first helicase like domain (H1). Gives cold sensitive phenotype (Raghunathan and Guthrie, 1998). Extracts containing this *brr2* mutant contain low levels of tri-snRNP and cannot support *in vitro* splicing (Raghunathan and Guthrie, 1998). This mutation causes a defect in U4/U6 unwinding (Raghunathan and Guthrie, 1998) and in disassembly of the post splicing complex (Small et al., 2006).
P841L	This study	Proline to leucine substitution in H1.
G873L	This study	Glycine to leucine substitution in H1. Mutation lies within an ATPase consensus sequence, QMLGRAGR.
E909K	Xu et al., 1996	Glutamic acid to lysine substitution in H1.Reported to be temperature sensitive above 33°C, and have a slow growth phenotype at temperatures between 23 and 30°C (Xu et al., 1996). *In vitro* splicing assays revealed that this mutation causes a block at, or before, the first step of splicing (Xu et al., 1996).
N1104L	Zhao et al., 2009	Asparagine to leucine substitution in S1. This is the yeast equivalent of a human mutation causing autosomal-dominant Retinitis Pigmentosa (Zhao et al., 2009). This mutation causes a defect in U4/U6 unwinding (Zhao et al., 2009).
R1107A	Small et al., 2006	Arginine to alanine substitution in S1. This mutation causes a cold sensitive phenotype, and defect in release of excised introns and dissociation of snRNAs (Small et al., 2006).
R1107L	Li et al., 2006	Arginine to leucine substitution in S1. This is the yeast equivalent of a human mutation causing autosomal-dominant Retinitis Pigmentosa (Zhao et al., 2009). This mutation leads to a cold sensitive phenotype at 15°C and a defect in U4/U6 unwinding (Zhao et al., 2009).
F1149I	This study	Phenylalanine to isoleucine substitution in S1.
G1375D,K1376N	Kim and Rossi, 1999	Glycine to aspartic acid and lysine to asparagine substitutions in the second helicase domain (H2). Changes the highly conserved ATPase motif, GKT, to DNT (Kim and Rossi, 1999).
D1474G	Kim and Rossi, 1999	Aspartic acid to glycine substitution in H2. Changes the highly conserved ATPase motif, DDAH, to GDAH (Kim and Rossi, 1999).

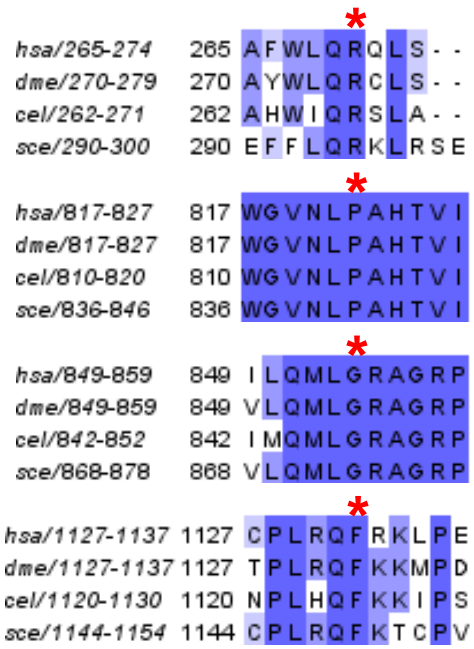


Figure 4.16 Conserved residues of Brr2p chosen for mutagenesis. This figure shows an alignment of the amino acid sequence of regions of Brr2p from *Homo sapiens* (hsa), *Drosophila melanogaster* (dme), *Caenorhabditis elegans* (cel) and *Saccharomyces cerevisiae* (sce). The figure illustrates the conserved nature of amino acids R295, P841, G873 and F1149 that were mutated creating novel Brr2p mutants. Mutated amino acids are highlighted with a red asterisk. Amino acids conserved in all species are highlighted in dark blue, those conserved in three of the species are highlighted in medium blue and those conserved in two out of the four species are highlighted in pale blue. Alignment was produced using ClustalW and Jal view.

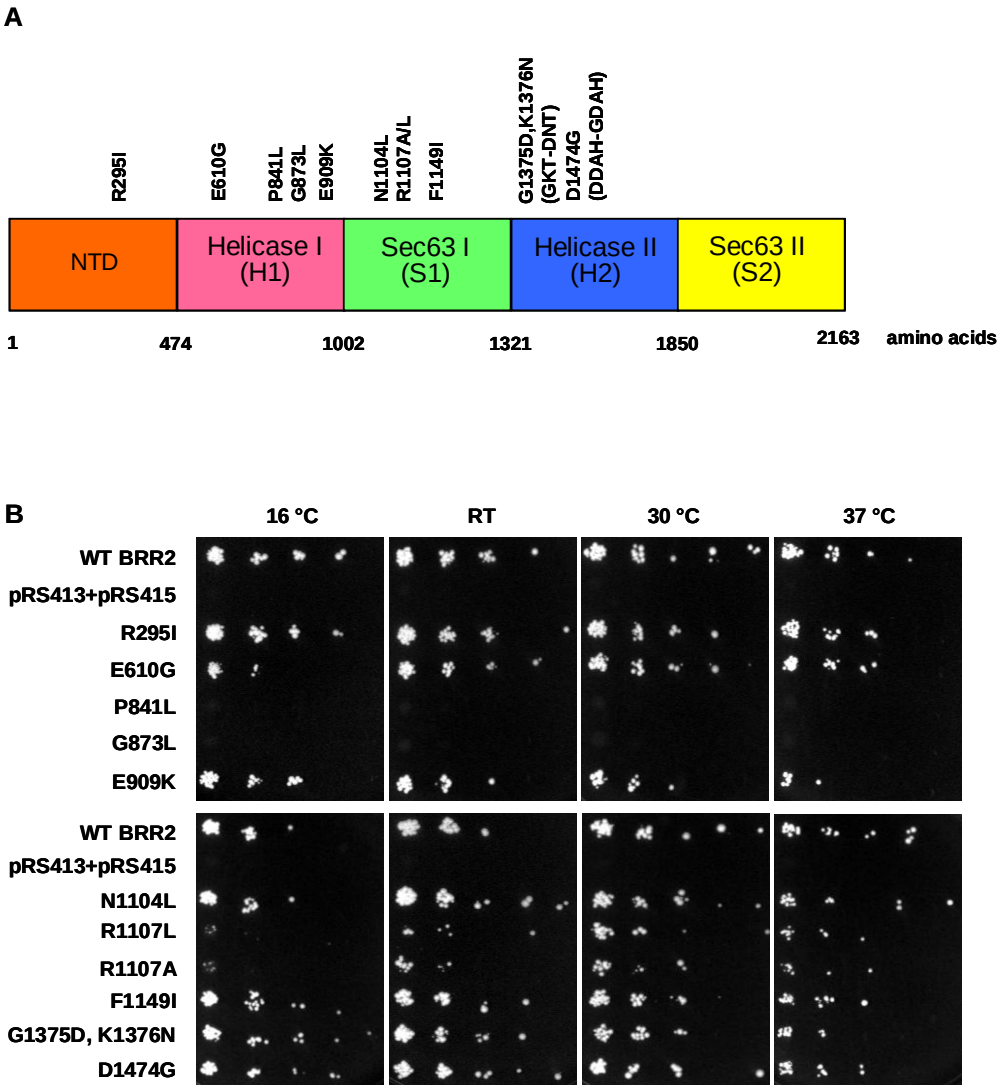


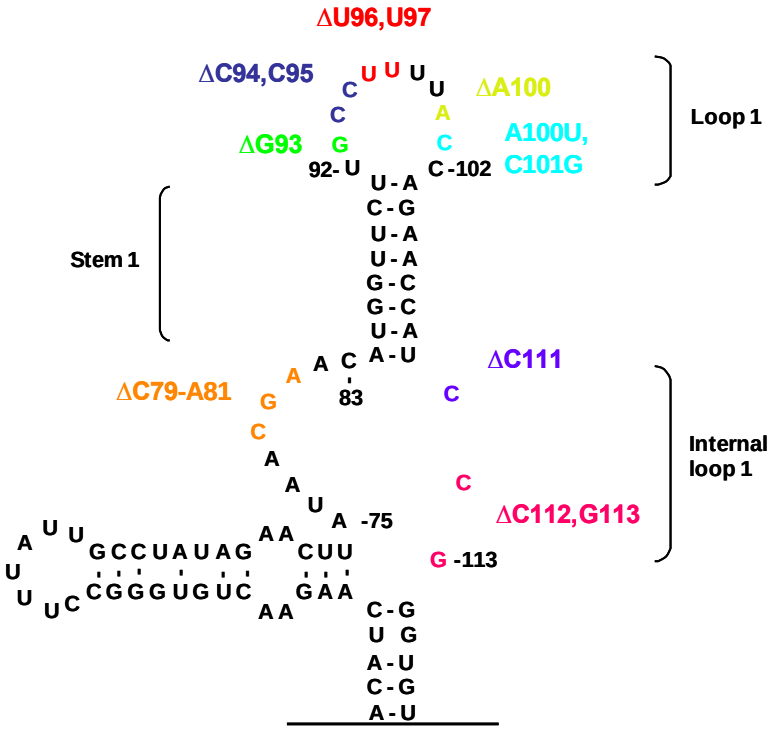
Figure 4.17 Eleven different *brr2* mutants for use in genetic screens. A. Four novel and seven previously published *brr2* mutants were constructed to investigate synthetic lethal interactions with U5 snRNA (see Table 4.1 for details). The four novel *brr2* mutants contain substitutions of conserved amino acids in Brr2p. The diagram illustrates the positions of the mutations in Brr2p. The N-terminal domain (NTD), first and second helicase-like domains (H1 and H2) and the two Sec63 domains (S1 and S2) of Brr2p are also indicated on this diagram. B. The eleven *brr2* mutants were tested for viability via a plasmid shuffle assay, in a *BRR2*/U5 snRNA deletion strain, in the presence of wild-type U5. A 1 in 5 serial dilution was carried out starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. On each plate a positive control strain containing wild-type *BRR2* and U5, and a negative control strain containing pRS413 and pRS415 were also present. Spot plates were incubated at 16°C, room temperature (RT), 30°C and 37°C.

and the viability of the two H2 mutants supports the hypothesis that it is the first helicase domain that functions in the essential process of U4/U6 unwinding (Kim and Rossi, 1999).

To investigate genetic interactions between *BRR2* and U5 snRNA, a synthetic lethal screen was carried out using the viable *brr2* mutants and a set of U5 snRNA mutants (Figure 4.18A). The U5 snRNA mutants chosen for use in the screen were U5 ΔC79-A81, containing a deletion in the 5' side of U5 snRNA IL1, two mutants containing deletions in the 3' side of U5 snRNA IL1, ΔC111 and ΔC112,G113, and several mutants with mutations in U5 Loop1. These U5 snRNA mutants were ΔG93, ΔC94,C95, ΔU96,U97 and ΔA100 deletion mutants and A100U,C101G substitution mutant. U5 snRNAs containing mutations in the Internal Loop 1 (IL1) and Loop 1 of U5 snRNA were selected for use in this screen because Snu114p is known to crosslink to IL1, and Prp8p is known to crosslink to both IL1 and Loop 1 (Dix et al., 1998). Snu114p has also been shown to have genetic interactions with Loop 1 and IL1 of U5 snRNA (Frazer et al., 2009).

Prior to use in the genetic screen, the U5 snRNA mutants were tested for viability with out any *brr2* mutants present, in combination with wild-type *BRR2*, at 16°C, RT, 30°C and 37°C (Figure 4.18B). The U5 snRNA Δ79-81 mutant was viable at all temperatures tested. Of the U5 snRNA loop 1 mutants used in the screen, ΔG93, A100,C101G and ΔA100 were viable at all temperatures tested. The U5 ΔC94,C95 was sick at 16°C, showing reduced growth at this temperature, viable at RT and 30°C, and lethal 37°C. The U5 ΔU96,U97 was viable at 16°C, RT and 30°C, and lethal at 37°C. Of the two U5 snRNA mutants carrying deletions in the 3' side of IL1, ΔC111 were viable at 16°C, RT and 30°C and sick at 37°C showing reduced growth at this temperature, whereas ΔC112,G113 was lethal at 16 and 37°C, and sick at RT and 30°C, with slow growth at these temperatures. All of these U5 snRNA mutants have been

A


ΔU96,U97
ΔC94,C95
ΔA100
ΔG93
A100U,
C101G
Loop 1
Stem 1
ΔC111
ΔC79-A81
Internal
loop 1
ΔC112,G113
92- U
C -102
83
-75
G -113

B

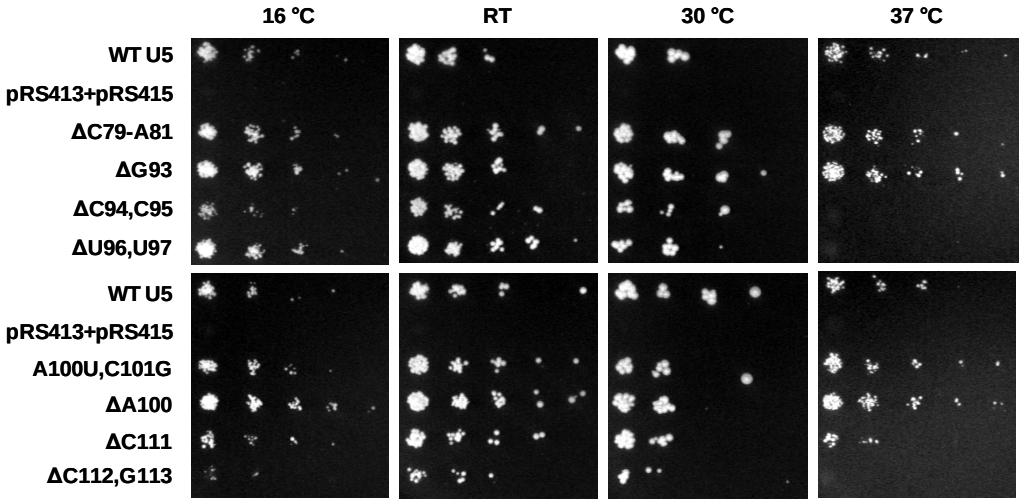

16 °C
RT
30 °C
37 °C
WT U5
pRS413+pRS415
ΔC79-A81
ΔG93
ΔC94,C95
ΔU96,U97
WT U5
pRS413+pRS415
A100U,C101G
ΔA100
ΔC111
ΔC112,G113

Figure 4.18 *In vivo* analysis of U5 snRNA mutants utilised for genetic screens. A. The eight U5 snRNA mutants used in the genetic screen to investigate interactions with *BRR2*. These mutations are highlighted on the diagram of the upper section of U5 snRNA, including Loop 1, Stem 1 and Internal Loop 1. B. The eight U5 snRNA mutants were tested for viability in the presence of wild-type *BRR2*. A 1 in 5 serial dilution was carried out starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. On each plate a positive control strain containing wild-type *BRR2* and U5, and a negative control strain containing pRS413 and pRS415 were also present. Spot plates were incubated at 16°C, room temperature (RT), 30°C and 37°C.

previously published, and have all been shown to be viable at 30°C (Frazer et al., 2009; O'Keefe and Newman, 1998).

A genetic screen was carried out, testing every combination of viable *brr2* mutant and U5 snRNA mutant via plasmid shuffle (Figure 4.19 to 4.23). Of all 72 combinations tested, four synthetic lethal interactions were found (Figure 4.19 to 4.23). These were all with U5-ΔC112,G113. Brr2-R295I and E610G were both lethal with U5-ΔC112,G113 at room temperature. Brr2-R1107A and R1107L were both lethal with U5-ΔC112,G113 at 30°C. These results would suggest that the N-terminal domain, first helicase like domain and first Sec63 domain of Brr2p are involved in associations with U5 snRNA.

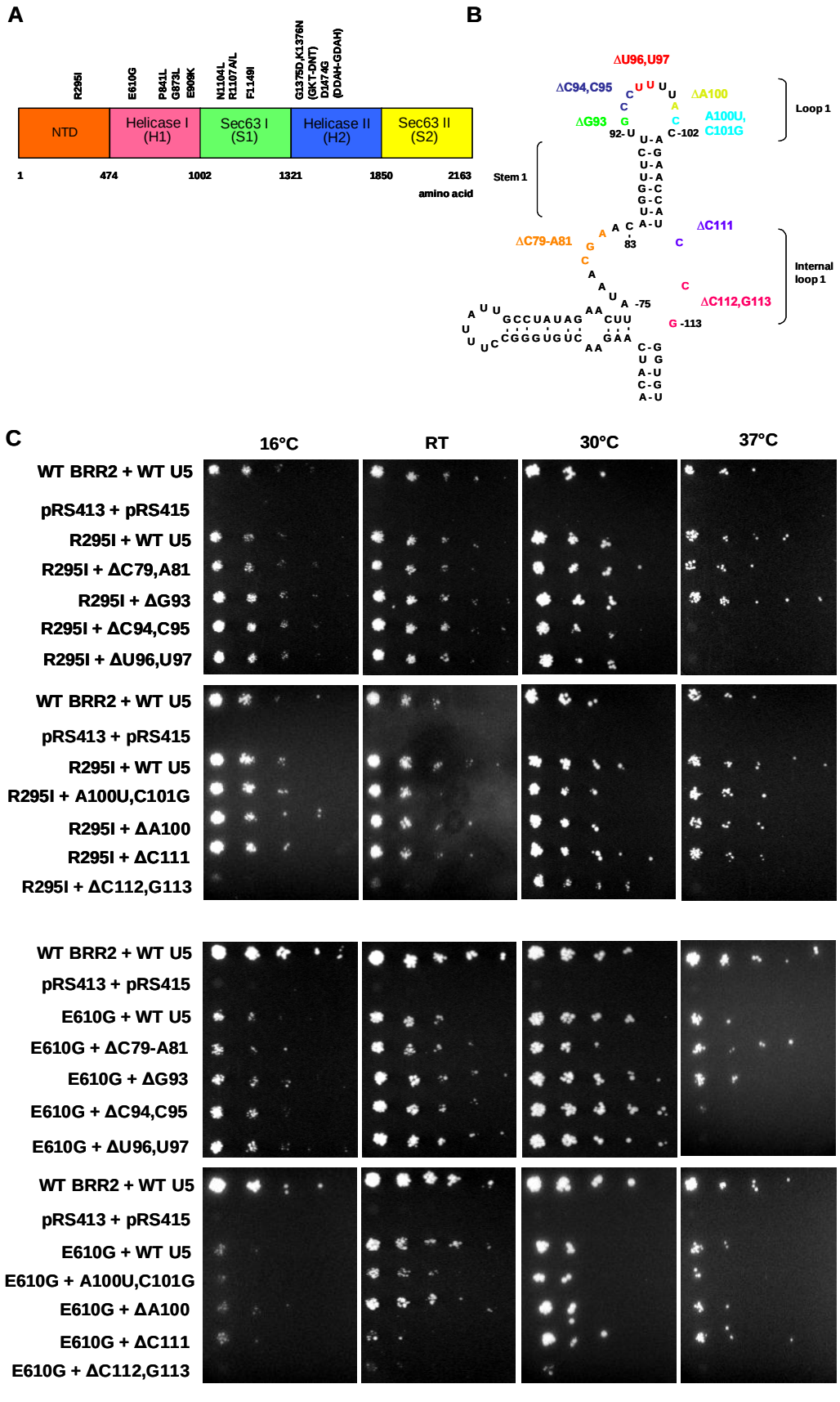

A
R295I
E610G
P841L
G873L
E909K
N1104L
R1107A/L
F1149I
G1375D,K1376N
(GKT-DNT)
D1474G
(DDAH-GDAH)
NTD
Helicase I
(H1)
Sec63 I
(S1)
Helicase II
(H2)
Sec63 II
(S2)
1
474
1002
1321
1850
2163
amino acid
B
ΔU96,U97
ΔC94,C95
ΔA100
ΔG93
A100U,
C101G
Loop 1
Stem 1
ΔC111
ΔC79-A81
Internal
loop 1
ΔC112,G113
C
16°C
RT
30°C
37°C
WT BRR2 + WT U5
pRS413 + pRS415
R295I + WT U5
R295I + ΔC79,A81
R295I + ΔG93
R295I + ΔC94,C95
R295I + ΔU96,U97
WT BRR2 + WT U5
pRS413 + pRS415
R295I + WT U5
R295I + A100U,C101G
R295I + ΔA100
R295I + ΔC111
R295I + ΔC112,G113
WT BRR2 + WT U5
pRS413 + pRS415
E610G + WT U5
E610G + ΔC79-A81
E610G + ΔG93
E610G + ΔC94,C95
E610G + ΔU96,U97
WT BRR2 + WT U5
pRS413 + pRS415
E610G + WT U5
E610G + A100U,C101G
E610G + ΔA100
E610G + ΔC111
E610G + ΔC112,G113

Figure 4.19 Genetic interactions between the *brr2* mutants R295I and E610G, and eight U5 snRNA mutants. Brr2-R295I and Brr2-E610G (A) were tested for genetic interactions with eight U5 snRNA mutants (B), by plasmid shuffle (C). The assay was performed in a *BRR2*/U5 deletion strain, carrying the wild-type genes on a *URA3* plasmid, which was co-transformed with different combinations of *brr2* and U5 mutants. A 1 in 5 serial dilution was carried out, starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. A positive control strain containing wild-type *BRR2* and U5, and a negative control strain containing pRS413 and pRS415 were also spotted onto each plate. Spot plates were incubated at 16 °C, room temperature (RT), 30 °C and 37 °C.

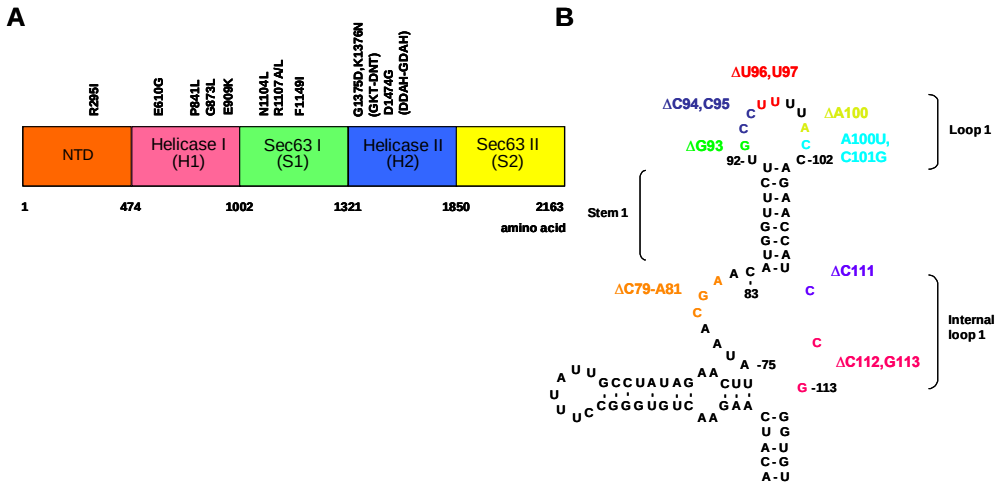
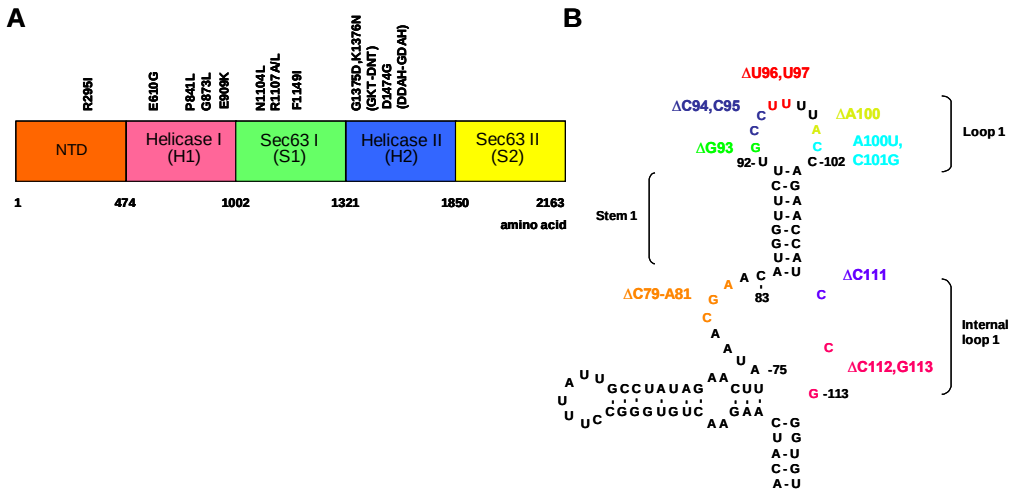

A
R295I
E610G
P841L
G873L
E909K
N1104L
R1107A/L
F1149I
G1375D,K1376N
(GKT-DNT)
D1474G
(DDAH-GDAH)
NTD
Helicase I (H1)
Sec63 I (S1)
Helicase II (H2)
Sec63 II (S2)
1
474
1002
1321
1850
2163
amino acid
B
ΔU96,U97
ΔC94,C95
ΔA100
ΔG93
A100U, C101G
Loop 1
Stem 1
ΔC79-A81
ΔC111
Internal loop 1
ΔC112,G113
C
16°C
RT
30°C
37°C
WT BRR2 + WT U5
pRS413 + pRS415
E909K + WT U5
E909K +ΔC79-A81
E909K + ΔG93
E909K + ΔC94,C95
E909K + ΔU96,U97
WT BRR2 + WT U5
pRS413 + pRS415
E909K + WT U5
E909K + A100U,C101G
E909K + ΔA100
E909K + ΔC111
E909K + ΔC112,G113
WT BRR2 + WT U5
pRS413 + pRS415
N1104L + WT U5
N1104L + ΔC79-A81
N1104L + ΔG93
N1104L + ΔC94,C95
N1104L + ΔU96,U97
WT BRR2 + WT U5
pRS413 + pRS415
N1104L + WT U5
N1104L + A100U,C101G
N1104L + ΔA100
N1104L + ΔC111
N1104L + ΔC112,G113

Figure 4.20 Genetic interactions between the *brr2* mutants E909K and N1104L, and eight U5 snRNA mutants. Brr2-E909K and Brr2-N1104L (A) were tested for genetic interactions with eight U5 snRNA mutants (B), by plasmid shuffle (C). The assay was performed in a *BRR2*/U5 deletion strain, carrying the wild-type genes on a *URA3* plasmid, which was co-transformed with different combinations of *brr2* and U5 mutants. A 1 in 5 serial dilution was carried out, starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. A positive control strain containing wild-type *BRR2* and U5, and a negative control strain containing pRS413 and pRS415 were also spotted onto each plate. Spot plates were incubated at 16 °C, room temperature (RT), 30 °C and 37 °C.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E610G
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G873L
E909K
N1104L
R1107A/L
F1149I
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DJ474G
(DDAH-GDAH)
NTD
Helicase I
(H1)
Sec63 I
(S1)
Helicase II
(H2)
Sec63 II
(S2)
1
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1002
1321
1850
2163
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ΔC94,C95
ΔA100
ΔG93
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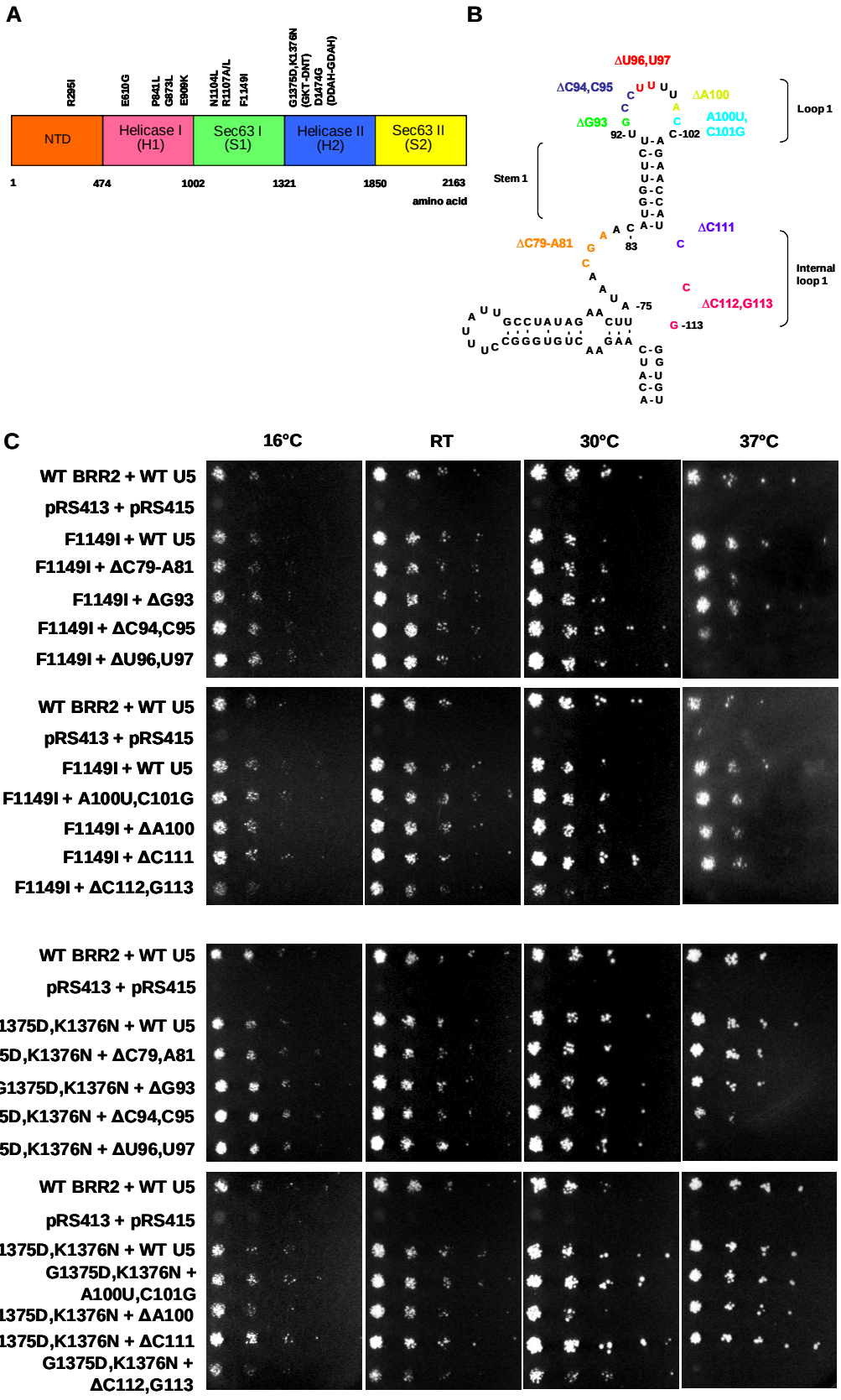
C101G
Loop 1
Stem 1
ΔC111
ΔC79-A81
Internal
loop 1
ΔC112,G113
C
16°C
RT
30°C
37°C
WT BRR2 + WT U5
pRS413 + pRS415
R1107A + WT U5
R1107A + ΔC79-A81
R1107A + ΔG93
R1107A + ΔC94,C95
R1107A + ΔU96,U97
WT BRR2 + WT U5
pRS413 + pRS415
R1107A + WT U5
R1107A + A100U,C101G
R1107A + ΔA100
R1107A + ΔC111
R1107A + ΔC112,G113
WT BRR2 + WT U5
pRS413 + pRS415
R1107L + WT U5
R1107L + ΔC79-A81
R1107L + ΔG93
R1107L + ΔC94,C95
R1107L + ΔU96,U97
WT BRR2 + WT U5
pRS413 + pRS415
R1107L + WT U5
R1107L + A100U,C101G
R1107L + ΔA100
R1107L + ΔC111
R1107L + ΔC112,G113

Figure 4.21 Genetic interactions between the *brr2* mutants, R1107A and R1107L, and eight U5 snRNA mutants. Brr2-R1107A and Brr2-R1107L (A) were tested for genetic interactions with eight U5 snRNA mutants (B), by plasmid shuffle (C). The assay was performed in a *BRR2*/U5 deletion strain, carrying the wild-type genes on a *URA3* plasmid, which was co-transformed with different combinations of *brr2* and U5 mutants. A 1 in 5 serial dilution was carried out, starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. A positive control strain containing wild-type *BRR2* and U5, and a negative control strain containing pRS413 and pRS415 were also spotted onto each plate. Spot plates were incubated at 16 °C, room temperature (RT), 30 °C and 37 °C.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R295I
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N1104L
R1107A/L
F1149I
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D1474G
(DDAH-GDAH)
NTD
Helicase I
(H1)
Sec63 I
(S1)
Helicase II
(H2)
Sec63 II
(S2)
1
474
1002
1321
1850
2163
amino acid
B
ΔU96,U97
ΔC94,C95
ΔA100
ΔG93
A100U,
C101G
Loop 1
Stem 1
ΔC111
ΔC79-A81
Internal
loop 1
ΔC112,G113
C
16°C
RT
30°C
37°C
WT BRR2 + WT U5
pRS413 + pRS415
F1149I + WT U5
F1149I + ΔC79-A81
F1149I + ΔG93
F1149I + ΔC94,C95
F1149I + ΔU96,U97
WT BRR2 + WT U5
pRS413 + pRS415
F1149I + WT U5
F1149I + A100U,C101G
F1149I + ΔA100
F1149I + ΔC111
F1149I + ΔC112,G113
WT BRR2 + WT U5
pRS413 + pRS415
G1375D,K1376N + WT U5
G1375D,K1376N + ΔC79,A81
G1375D,K1376N + ΔG93
G1375D,K1376N + ΔC94,C95
G1375D,K1376N + ΔU96,U97
WT BRR2 + WT U5
pRS413 + pRS415
G1375D,K1376N + WT U5
G1375D,K1376N +
A100U,C101G
G1375D,K1376N + ΔA100
G1375D,K1376N + ΔC111
G1375D,K1376N +
ΔC112,G113

Figure 4.22 Genetic interactions between the *brr2* mutants, F1149I and G1375D,K1376N, and eight U5 snRNA mutants. Brr2-F1149I and Brr2-G1375D,K1376N (A) were tested for genetic interactions with eight U5 snRNA mutants (B), by plasmid shuffle (C). The assay was performed in a *BRR2*/U5 deletion strain, carrying the wild-type genes on a *URA3* plasmid, which was co-transformed with different combinations of *brr2* and U5 mutants. A 1 in 5 serial dilution was carried out, starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. A positive control strain containing wild-type *BRR2* and U5, and a negative control strain containing pRS413 and pRS415 were also spotted onto each plate. Spot plates were incubated at 16 °C, room temperature (RT), 30 °C and 37 °C.

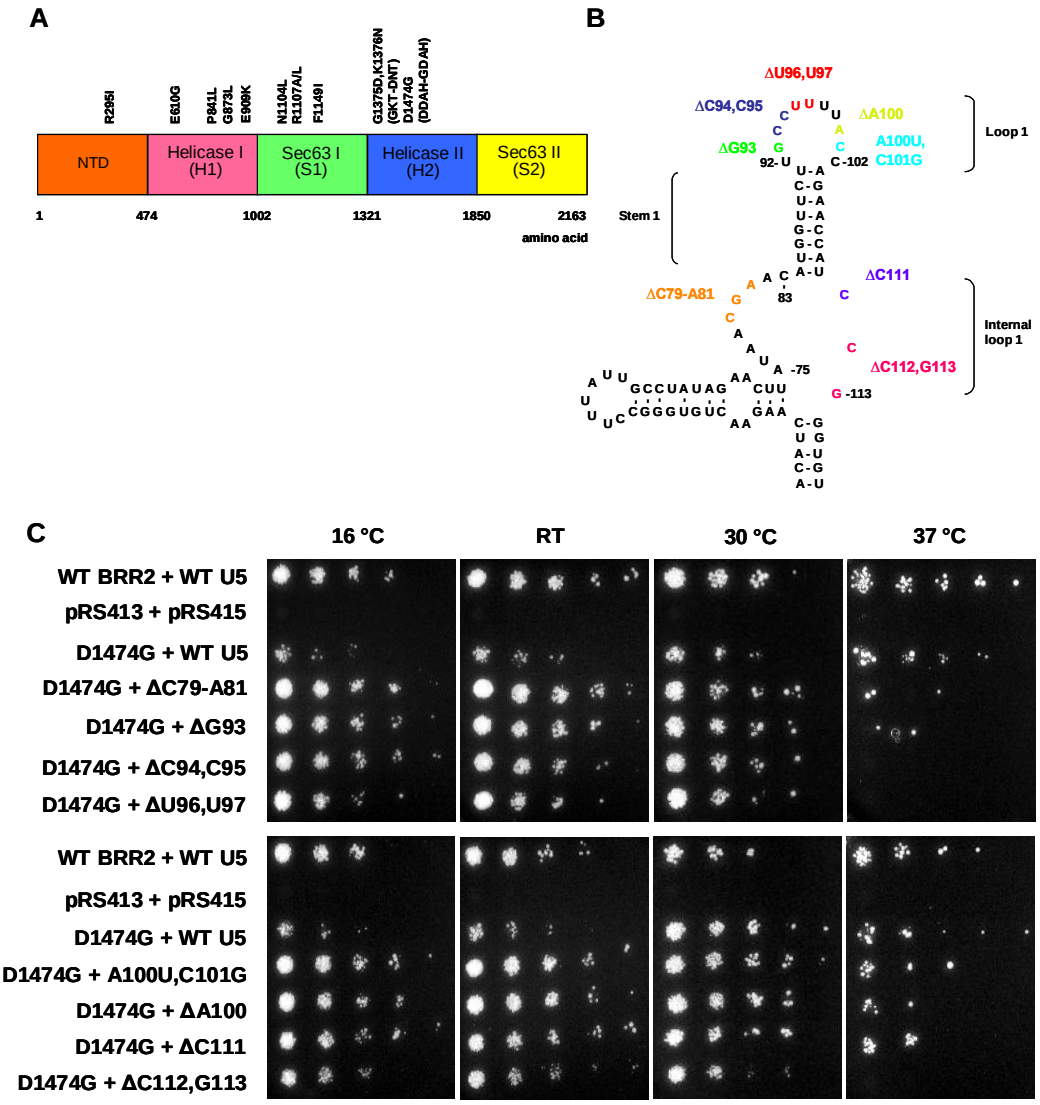

Figure 4.23 Genetic interactions between the *brr2* mutant D1474G and eight U5 snRNA mutants. Brr2-D1474G (A) was tested for genetic interactions with eight U5 snRNA mutants (B), by plasmid shuffle (C). The assay was performed in a *BRR2*/U5 deletion strain, carrying the wild-type genes on a *URA3* plasmid, which was co-transformed with different combinations of *brr2* and U5 mutants. A 1 in 5 serial dilution was carried out, starting at OD 600 of 1. Each dilution was spotted onto 5FOA containing plates. A positive control strain containing wild-type *BRR2* and U5, and a negative control strain containing pRS413 and pRS415 were also spotted onto each plate. Spot plates were incubated at 16 °C, room temperature (RT), 30 °C and 37 °C.

4.2 Discussion

The spliceosome is a large protein RNA complex, in which many specifically timed and orientated protein-protein, protein-RNA and RNA-RNA interactions must take place to facilitate splicing. The U5 snRNP is a major spliceosome component which is present in the active spliceosome and is required for both steps of pre-mRNA splicing. The U5 snRNP is comprised of the U5 snRNA associated with seven Sm proteins and approximately six U5 snRNP specific proteins, including the core U5 snRNP proteins, Brr2p, Snu114p and Prp8p (Fabrizio et al., 2009). Correct assembly of the U5 snRNP is a very important process, and is essential for formation of the U4/U6.U5 tri-snRNP complex and subsequent assembly of the pre-catalytic spliceosome. The U5 snRNP, in particular Prp8p and Loop 1 of the U5 snRNA, contribute to the catalytic core of the spliceosome (Abelson, 2008; Newman, 1997). However, little is known about assembly of the U5 snRNP and the U4/U6.U5 tri-snRNP. To increase understanding and knowledge of interactions that take place between Brr2p, Snu114p and Prp8p, and the U5 snRNA, and the U5 snRNA requirements for association of these proteins in U5 snRNP formation, immunoprecipitations and genetic screening were carried out. This study has supported the importance of IL1 and Loop 1 of the U5 snRNA in associations of U5 snRNP proteins. Due to differences seen in effects of U5 mutations on association of Brr2p, Snu14p and Prp8p, these data also indicated that, despite the intimate interactions between Brr2p, Snu114p and Prp8p, they do appear to have some interactions with the U5 snRNA independent of each other and do not just bind to the U5 snRNA as a tri-protein complex.

4.2.1 Interactions of the U5 snRNP proteins Brr2p, Snu114p and Prp8p with U2, U4, U5 and U6 snRNAs

To determine which snRNAs the U5 snRNP proteins, Brr2p, Snu114p and Prp8p associate with in yeast whole cell extracts, immunoprecipitations were performed. It was revealed that Brr2p, Snu114p and Prp8p not only associate with U5 snRNA, but also associate with U2, U4 and U6 snRNA (Figures 4.1 to 4.3).

The U5 snRNP is present as a single particle, as a component of the U4/U6.U5 tri-snRNP, and as part of the fully assembled pre-catalytic spliceosome or penta-snRNP. Following release of U1 and U4 snRNPs, the U5 snRNP is also present in the catalytically active spliceosome with the U2 and U6 snRNPs (Staley and Guthrie, 1998). However, as immunoprecipitations in this study were carried out in yeast whole cell extracts, without subjecting them to splicing conditions and without an excess of pre-mRNA substrate, it is expected that only U5 snRNA in the U5 snRNP and U4/U6.U5 tri-snRNP particles, will be immunoprecipitated and not pre-catalytic and catalytically active spliceosomes. The fact that Brr2p, Snu114p and Prp8p all immunoprecipitated the U4, U5 and U6 snRNAs, would suggest that these proteins are assembled in the U5 snRNP and U4/U6.U5 tri-snRNP particles in yeast whole cell extracts. Brr2p, Snu114p and Prp8p also associated with the U2 snRNA in whole cell yeast extracts. Three U4/U6.U5 tri-snRNP associated proteins, Snu66p, Snu23p and Spp381p, that have also been shown to immunoprecipitate U2 snRNA (Gottschalk et al., 1999). Therefore, the associations seen between Brr2p, Snu114p and Prp8p with the U2 snRNA are most probably indirect associations, with Snu66p, Snu23p and Spp381p providing the link between the tri-snRNP and U2 snRNP (Gottschalk et al., 1999). The NTC protein Cwc21p could be also involved in indirect associations of Snu114p and Prp8p with the U2 snRNA. Cwc21p has been shown to interact with the N-terminus of Prp8p and the C-terminus

of Snu114p, and also associates with U2 snRNP proteins (Grainger et al., 2009; Khanna et al., 2009). This presents a second possible explanation for the association seen between U5 snRNP proteins and the U2 snRNA.

These finding support the proposed presence of a U2.U5.U4/U6 tetra-snRNP (Gottschalk et al., 1999; Raghunathan and Guthrie, 1998). It has been suggested that a pre-formed tetra-snRNP complex may be an intermediate phase in the spliceosome assembly and activation pathway (Gottschalk et al., 1999). It is possible that the U2 snRNP associated proteins that also associate with the tri-snRNP may have a role in the tri-snRNP complex joining the spliceosome (Gottschalk et al., 1999).

To investigate interactions of Brr2p, Snu114p and Prp8p with snRNAs within pre-catalytic or catalytically active spliceosomes, rather than in U5 snRNP and U4/U6.U5 tri-snRNP particles, yeast whole cell extracts could be incubated under splicing conditions prior to immunoprecipitations. Pre-mRNA substrates could be used that stall the spliceosome at various steps of splicing. Actin pre-mRNA substrate can be cleaved at a ClaI site within the intron just down stream from the branch point to stall spliceosomes prior to the first step of splicing (Cheng, 1994). Actin pre-mRNA substrate containing an AG to AC mutation at the 3' splice site can be used to stall spliceosomes prior to the second step of splicing (Cheng, 1994). Spliceosomes can also be arrested using protein manipulation. For example depletion of Prp16p causes a block at the second step of splicing and Prp22p mutants, such as *prp22*-D603A, cause a block in splicing after the second step but before disassembly of the post splicing complex (Schneider et al., 2002). This allows accumulation and analysis of, complexes formed at specific stages of pre-mRNA splicing.

Data here suggest that the U5 snRNP proteins, Brr2p, Snu114p and Prp8p do not interact with the U1 snRNP in yeast whole cell extracts. Although U1 and U5 snRNP particles are both present in fully assembled pre-catalytic spliceosomes, these immunoprecipitations were not carried out under splicing conditions, so fully assembled spliceosomes are unlikely to be present.

Although interactions between Brr2p, Snu114p and Prp8p with U2, U4, U5 and U6 snRNAs in yeast whole cell extracts have been demonstrated here, these interactions may not be direct. It is possible that Brr2p, Snu114p and Prp8p, the human homologues of which are able to form a stable RNA-free complex (Achsel et al., 1998), interact with U2, U4, U5 and U6 snRNAs indirectly. However, it is known that Snu114p crosslinks to U5 snRNA and Prp8p crosslinks to U5 and U6 snRNAs, which indicates that these protein-RNA interactions are direct (Dix et al., 1998; Vidal et al., 1999).

Work on the human homologues of Brr2p, Snu114p and Prp8p (hBrr2p, hSnu114p and hPrp8p) revealed extensive interactions between these proteins, and has demonstrated that these proteins can form a stable RNA-free complex (Achsel et al., 1998; Liu et al., 2006). The N and C-termini of hPrp8p interact with both hBrr2p and hSnu114p, and the second helicase domain of Brr2p interacts with Snu114p (Liu et al., 2006). An intramolecular fold within Prp8p has also been shown to bind to the N-terminal half of Snu114p (Grainger et al., 2009). It is also known that Prp8p can crosslink to the U6 snRNA, so it is possible that the association of Prp8p with U4 snRNA is via the U6 snRNA, which is extensively base-paired with U4 snRNA in the U4/U6.U5 tri-snRNP (Hashimoto and Steitz, 1984). Therefore, it is also possible that the associations of Brr2p with U2, U4, U5 and U6 snRNAs, and of Snu114p with U2, U4 and U6 snRNAs, are indirect, via the extensive interactions of Brr2p and Snu114p with Prp8p (Achsel et al., 1998; Grainger et al., 2009; Liu et al., 2006).

Indirect interactions of these proteins and snRNAs, does seem likely, but it is possible that some of the interactions seen here are in fact direct. With Brr2p and Snu114p being involved in U4/U6 unwinding and disassembly of the post-splicing complex, it is possible that Brr2p and/or Snu114p interact directly with U2, U4 or U6 snRNAs during RNA unwinding required for U4 release and probably U2/U6 unwinding during disassembly of the post-splicing complex. To test for direct interactions between Brr2p, Snu114p and Prp8p with snRNAs in yeast whole cell extracts, crosslinking could be carried out prior to immunoprecipitations, and indirect interactions would be dissociated using high salt washes. This means that only direct interactions would be detected. The identification of direct protein-RNA interactions would further increase understanding of the functions of Brr2p, Snu114p and Prp8p. For example a direct interaction of Brr2p with the U2 snRNA would support a direct role in Brr2p unwinding of the U2/U6 helices, and direct interactions of Snu114p with U4 or U6 would support a role for Snu114p in detecting the state of the U4/U6 helix and a role in controlling the unwinding activities of Brr2p.

Aside from the published crosslinking data regarding interactions between Snu114p and Prp8p with the U5 and U6 snRNAs, several studies have identified genetic interactions between Snu114p and Prp8p with these and other snRNAs. Genetic interactions between Snu114p and U2, U4, U5 and U6 snRNA, between Prp8p and U4 and U6 snRNAs, and between Brr2p and the U2 and U6 snRNAs have been identified (Frazer et al., 2009; Kuhn and Brow, 2000; Kuhn et al., 2002; Xu et al., 1998; Xu et al., 1996). Although these genetic interactions may not represent direct protein-RNA interactions, it does emphasise the complex functional network of interactions, involving Snu114p, Brr2p and Prp8p, within the spliceosome, required for the execution and control of several major events in pre-mRNA splicing.

4.2.2 Viability of U5 snRNA mutants *in vivo*

Although it is known that the U5 snRNP and U4/U6.U5 tri-snRNP particles are essential for recognition of the pre-mRNA and assembly of the spliceosome for pre-mRNA splicing, little is known about how these particles are formed. To increase our knowledge of how proteins associate with the U5 snRNA to form the U5 snRNP, and contribute to the catalytic core of the spliceosome, a set of U5 snRNA mutants was designed and tested for viability. To allow investigations using lethal U5 snRNA mutants, wild-type U5 snRNA must be present to enable growth of yeast cells. Several U5 snRNA mutants tested in this study were lethal, so U5 snRNA mutants were constructed in a plasmid in which U5 snRNA contained a 20 nucleotide insert. The 20 nucleotide insert allows wild-type and mutant U5 snRNA to be differentiated between by size difference when they are both present. The insert does not affect the function of the U5 snRNA or the viability of cells (Figure 4.5).

Several U5 snRNA mutants were constructed and tested for viability. Deletion of the 5' side of U5 snRNA IL1 (Δ75-83) caused a lethal phenotype, which was expected as deletion of this region has been previously shown to be lethal (Figure 4.6) (Frank et al., 1994). The sequence substitution of the 5' side of U5 snRNA IL1 was also lethal (Figure 4.6). In both cases, it is possible that the lethal phenotype is due to disruption in the secondary structure of U5 snRNA. The sequence of the 5' side of U5 snRNA IL1 is only moderately conserved, with the only invariant nucleotides being C79, to which Snu114p and Prp8p crosslink, and G80 (Dix et al., 1998; Frank et al., 1994). Deletion of nucleotides 79 to 80 and 78 to 81 in the 5' side of U5 snRNA IL1 has previously been shown to cause no lethal phenotype at 30°C (Frazer et al., 2009). In this study these mutants were also tested at 16°C, RT and 37°C. Due to the C79,G80 dinucleotide being invariant in all U5 species (Frank et al., 1994), it was surprising that deletion of nucleotides 78 to 81 (Δ78-80) and 79 to 80 (Δ79-80), from the 5'

side of U5 snRNA IL1 did not result in a growth phenotype at any temperature tested (Figure 4.6). This would suggest that the 5' side of U5 snRNA IL1 is resilient to deletions, with deletion of almost half of the loop not causing a lethal, or even sick phenotype. The 5' side of U5 snRNA IL1 is larger than the 3' side of IL1, so deletion of nucleotides in the 5' side may not affect the secondary structure of U5 snRNA, with formation of Stem 1 and the variable stem loop being unaffected. Deletion of the entire 5' side of IL1 would most probably effect the formation of stem 1 and the variable stem loop, and results in a lethal phenotype. These results also suggest that the function or purpose of the invariant CG dinucleotide must be dispensable, or that another region of U5 snRNA can carry out the role of nucleotides C79 and G80, in their absence. It is known that position C79 is involved in protein interactions with both Snu114p and Prp8p (Dix et al., 1998). It is possible that nucleotides C79 and G80 are both involved in protein interactions, but are not the only site of protein interaction, so proteins will still bind to the U5 snRNA in the absence of C79 and G80.

All of the deletions in U5 snRNA Loop 1 resulted in a lethal phenotype (Figure 4.6). This is most likely due to the importance of the loop and its conserved sequence in the alignment of exons for the second step of splicing (Frank et al., 1994; Newman and Norman, 1991; Newman and Norman, 1992; O'Keefe and Newman, 1998; O'Keefe et al., 1996). It is known that the first step of splicing can occur *in vitro* without U5 snRNA Loop 1 (O'Keefe et al., 1996). It is, however, known that the U5 snRNA Loop 1 is critical for alignment of exons for the second step, and that deletion of four nucleotides of U5 snRNA Loop 1 results in low crosslinking between Loop 1 and the 5' exon (O'Keefe and Newman, 1998). This is a possible reason for the lethal phenotype associated with the U5 snRNA Loop 1 mutations *in vivo*. It has also previously been shown that a mutation in U5 snRNA that reduces the size of Loop 1 effects the stability of Prp8p, thus effecting U5 snRNP and tri-snRNP assembly (Kershaw et

al., 2009). It is possible that the U5 snRNA Loop 1 mutants tested here are effecting Prp8p stability, thus effecting U5 snRNA and U4/U6.U5 tri-snRNP assembly and preventing splicing, resulting in a lethal phenotype. It is also thought that assembly of Snu114p into the U5 snRNP requires Prp8p (Brenner and Guthrie, 2006). So if Prp8p stability is being affected, assembly of Snu114p in to the U5 snRNP will also be affected.

All three deletions made in the 3' side of U5 snRNA IL1 resulted in a lethal phenotype at 30°C and 37°C, and sick phenotype at RT and 16°C (Figure 4.6). Deletion of nucleotides C111 to G113 of the 3' side of U5 snRNA IL1 has been shown in a previous study to be lethal (Frank et al., 1994). Deletion of nucleotide C111 of U5 snRNA has been shown to be viable at 30°C, however when tested here, resulted in a lethal phenotype at 30°C (Figure 4.6) (Frazer et al., 2009). This may be due to differences in yeast strain backgrounds. The U5 snRNA mutants, Δ111-113, Δ111-112 and Δ111, were all lethal at 30°C and 37°C (Figure 4.6). This may be due to the essential and highly conserved nature of the 3' side of U5 snRNA IL1, and also due to the small size of the 3' side of IL1. With the 3' side of U5 snRNA IL1 being made up of only three nucleotides, even a single nucleotide deletion could have a large effect on the secondary structure of U5 snRNA. Decreasing the size of the 3' side of U5 snRNA IL1 would shrink the region linking the 3' sides of Stem 1 and Stem 2, possible causing a kink in U5 snRNA or reduced flexibility of Stem 1 and Loop 1. This may affect how Loop 1 is positioned within the spliceosome and hinder Loop 1 positioning the exons at the catalytic core of the spliceosome for splicing to occur. The reason for the highly conserved nature of this region may be to maintain the secondary structure of the 3' side of U5 snRNA IL1.

When making mutations in RNA molecules it cannot be ruled out that lethal or sick phenotypes seen are due to disruption of the structure of the RNA. To test how the deletions made in the U5 snRNA affect the structure of U5 snRNA, structure probing by chemical

and/or enzymatic means could be carried out, and patterns compared to that of U5 with no mutation.

4.2.3 Associations of Brr2p, Snu114p and Prp8p with the U5 snRNA following mutation of the 5' side of IL1

The immunoprecipitations investigating the effects of deletions in the 5' side of U5 snRNA IL1, revealed that all deletions made in the 5' side of U5 snRNA IL1 affected associations of Brr2p, Snu114p and Prp8p with U5 snRNA, indicating that this region of U5 snRNA is involved in association of these proteins (Figures 4.7 to 4.9). Of the three mutants containing deletions in the 5' side U5 snRNA IL1, the largest deletion (Δ75-83) had the largest effect on association of each protein. The four nucleotide deletion (Δ78-81) did effect associations of Brr2p, Snu114p and Prp8p with U5 snRNA, but to a lesser extent than deletion of nucleotides 75 to 83. The two nucleotide deletion (Δ79-80) had the smallest effect on association of U5 snRNA with Brr2p, Snu114p and Prp8p (Figures 4.7 to 4.9B). This general trend, where effect on protein association is proportional to the size of deletion, with largest deletions having the largest effect, suggests that size of the 5' side of U5 snRNA IL1 is important for association of Brr2p, Snu114p and Prp8p. With this in mind the 5' side of U5 snRNA IL1 was substituted for the reverse complement of the wild-type sequence (75-83 sub), so the loop was the same size as in wild-type U5 snRNA, but the sequence was different. If only the size of the loop was important, then proteins may still associate with 75-83 sub U5 snRNA. However, Brr2p, Snu114p and Prp8p all showed either no, or very little, association with 75-83 sub U5 snRNA (Figures 4.7 to 4.9B). This indicates that either the sequence of the 5' side of U5 snRNA IL1 is important for associations of Brr2p, Snu114p and Prp8p, or that substitution of the 5' side of IL1 disrupted the structure of U5 snRNA, possibly by preventing or disrupting the formation

of the secondary structure of U5 snRNA. When substituting the sequence of the 5' side of IL1 nucleotide C83 at the base of stem 1 is substituted for a G. This G may form a base pair with nucleotide C111 of the 3' side of IL1, increasing the length of stem 1, and decreasing the size of both the 5' and 3' side of IL1. This could subsequently effect the association of Brr2p, Snu114p and Prp8p with the U5 snRNA and the structure of U5 snRNP. This possibility could be tested by substituting the 5' side of U5 snRNA IL1 with other sequences, to see if they all had the same effect, or by testing the structure of the mutant U5 snRNA by structural probing by chemical and/or enzymatic means, to determine how the mutation changes the structure compared to wild-type U5 snRNA.

Although the general trend of how the mutations in the 5' side of U5 snRNA IL1 influences associations of Brr2p, Snu114p and Prp8 was the same for each protein, the exact effects were not the same. The association of Snu114p with U5 snRNA was particularly sensitive to mutations in the 5' side of U5 snRNA IL1. Of the three proteins investigated, the association of Prp8p with U5 snRNA was least effected by mutations in the 5' side of U5 snRNA IL1 (Figures 4.7 to 4.9B). The large deletion (Δ75-83) and the sequence substitution (75-85 sub) practically abolished the association of mutant U5 snRNA with Snu114p, whereas Brr2p and Prp8p still showed some association with Δ75-83 and 75-83 sub U5 snRNA mutants (Figures 4.7 to 4.9B). This demonstrates that Brr2p and Prp8p can still associate with the U5 snRNA without Snu114p association of the U5 snRNA. This data also shows that the association of Snu114p with U5 snRNA is more sensitive to deletions in the 5' side of U5 snRNA IL1, than the association of Brr2p and Prp8p.

The fact that Prp8p and Snu114p still associate with the two U5 snRNA mutants containing smaller deletions, Δ78-81 and Δ79-80, is interesting because nucleotide C79, the site of both Prp8p and Snu114p crosslinking, is deleted in these mutants (Dix et al., 1998).

This suggests that the association of Prp8p and Snu114p with the 5' side of U5 snRNA IL1 is more extensive than previously demonstrated, and that position C79 is not the only sight anchoring these proteins to the U5 snRNA. Brr2p, Snu114p and Prp8p still associating with U5 snRNA lacking the invariant CG dinucleotide of the 5' side of U5 snRNA IL1, further supports the suggestion that this invariant dinucleotide is dispensable.

Where protein association is still seen with U5 snRNA mutants, it is possible that only one of the three proteins, Brr2p, Snu114p or Prp8p, is interacting with the U5 snRNA, and the other proteins are associating with the U5 snRNA indirectly, via interactions with that protein. The association of Snu114p with U5 snRNA was practically abolished with the U5 snRNA mutants Δ75-83 and 75-83 sub, but Prp8p and Brr2p still showed some association with the mutant U5 snRNA. It is not surprising that Prp8p still showed some association with the mutant U5 snRNAs, because Prp8p has been shown to crosslink to five different regions of U5 snRNA (Dix et al., 1998). Not only is the interaction between Prp8p and the U5 snRNA direct, but it is also extensive, so even in the absence of the nine nucleotides of the 5' side of U5 snRNA IL1, Prp8p could still associate with other regions of the U5 snRNA. Brr2p still associates with the U5 snRNA mutants Δ75-83 and 75-83 sub, so it is possible that Brr2p is binding the U5 snRNA indirectly, through protein-protein interactions with the C-terminus of Prp8p (Liu et al., 2006; van Nues and Beggs, 2001). However, as Snu114p association with the U5 snRNA mutants Δ75-83 and 75-83 sub, is so low, it suggests that protein-protein interactions between Snu114p and Prp8p are not sufficient for Snu114p association with these U5 snRNA mutants. This suggests that association of Snu114p with the U5 snRNA requires a direct interaction with the 5' side of U5 snRNA IL1. This hypothesis is supported by the fact that Snu114p does crosslink to the 5' side of U5 snRNA IL1 and synthetic sick interactions

identified between Snu114p and this region of U5 snRNA (Dix et al., 1998; Frazer et al., 2009).

4.2.4 Associations of Brr2p, Snu114p and Prp8p with the U5 snRNA following mutations of Loop 1

All of the mutant U5 snRNAs containing deletions in Loop 1 of U5 snRNAs affected association of Brr2p, Snu114p and Prp8p with U5 snRNA (Figures 4.10 to 4.12B). The largest deletion in U5 Loop 1, Δ92-102, had the largest effect on association of Brr2p, Snu114p and Prp8p with U5 snRNA (Figures 4.10 to 4.12B). The effect of this deletion on protein association seen in the case of Snu114p was not as large as the effect seen on association of Brr2p and Prp8p, suggesting that the association of Snu114p is less sensitive to deletions in Loop 1 of U5 snRNA. Snu114p still associating with U5 snRNA Δ92-102 indicates that, although Loop 1 is involved in association of Snu114p with U5 snRNA (as demonstrated by the reduction in Snu114p association with U5 snRNA Δ92-102), Snu114p must interacts with another region of U5 snRNA. It is likely that this region is the 5' side of IL1, and allows association of Snu114p with U5 snRNA in the absence of U5 snRNA Loop 1. The effects on Brr2p and Prp8p association with U5 snRNA with nucleotides 92 to 102 deleted were very similar. This supports the possibility that Brr2p may be interacting with U5 snRNA via Prp8p, which crosslinks extensively to the U5 snRNA (Dix et al., 1998).

The three smaller four nucleotide deletions (Δ92-95, Δ96-99 and Δ99-102), did not all have the same influence on associations of Brr2p, Snu114p and Prp8p with U5 snRNA. In the case of Brr2p, deletion of nucleotides 99-102 of U5 snRNA Loop 1 (Δ99-102) had the largest effect on associations with U5 snRNA, and deletion of nucleotides 96 to 99 of U5 snRNA loop 1 (Δ96-99), had the least effect (Figure 4.10). The fact that deletion of nucleotides 99 to

102 from U5 snRNA Loop 1 had more of an effect of Brr2p associations with U5 snRNA, than deletion of nucleotide 92 to 95 and 96 to 99, would suggest that of the nucleotides present in Loop 1, nucleotides 99 to 102 are most important for the association of Brr2p with the U5 snRNA. It is possible Brr2p binds preferentially to the 3' side of Loop1 of the U5 snRNA because it may position Brr2p in the correct location for U4/U6 unwinding within the tri-snRNP. It may also be possible that Snu114p and Prp8p are contacting the rest of the nucleotides in U5 Loop 1, so nucleotides 99 to 102 are most accessible for Brr2p association.

Of the smaller U5 snRNA Loop 1 deletions, Δ92-95 had the largest effect on associations of Snu114p with the U5 snRNA, and Δ99-102 had the smallest effect on association of Snu114p with the U5 snRNA with a reduction in association of only around 25% compared to association of U5 with no mutation (Figure 4.11). Deletion of nucleotides 96 to 99 of U5 Loop 1 had an intermediate effect on associations of Snu114p with U5 snRNA. The fact that Δ92-95 had the largest effect, Δ96-99 had an intermediate effect and Δ99 to 102 had the least effect on association of Snu114p with U5 snRNA would indicate that nucleotides in the 5' side of U5 Loop 1 are more important in the association of U5 snRNA with Snu114p, than nucleotides in the 3' half of Loop 1. With Snu114p crosslinking to the 5' side of IL1, these data suggest that Snu114p contacts the U5 snRNA down the 5' side of the secondary structure (Dix *et al*., 1998). With the suggestion that the 3' side of U5 Loop 1 is important for the association of Brr2p, it may be that if Brr2p is bound to the 3' side of Loop 1, only nucleotides in the 5' side of the Loop 1 are accessible for association of Snu114p.

All of the U5 snRNA mutants containing four nucleotide deletions in Loop 1 (Δ92-95, Δ96-99 and Δ99-102) reduced association of Prp8p by about 45 to 50% compared to association of Prp8p with U5 with no mutation (Figure 4.12). As each of the deletions of four nucleotides had a similar effect of association of Prp8p with U5 snRNA, it would suggest that

the size of Loop 1 of U5 snRNA is important for association of Prp8p. Prp8p has been shown to crosslink to position U97 in Loop 1 (Dix et al., 1998). The fact that Prp8p is still able to associate with U5 snRNA in the absence of nucleotide U97 demonstrates that this nucleotide is not essential for association of Prp8p.

4.2.5 Associations of Brr2p, Snu114p and Prp8p with the U5 snRNA following mutation of the 3' side of IL1

All of the deletions made in the 3' side of U5 snRNA IL1 resulted in large reductions in the amount of mutant U5 snRNA associating with Brr2p, Snu114p and Prp8p (Figures 4.13 to 4.15B). This indicates that the 3' side of U5 snRNA is important in associations of Brr2p, Snu114p and Prp8p with U5 snRNA. Deletions in the 3' side of U5 snRNA IL1 had slightly less effect on associations of U5 snRNA with Prp8p, than with Brr2p and Snu114p. It is known that Prp8p crosslinks to U5 snRNA Loop 1, both side of IL1 and both side IL2, so the interactions of Prp8p are extensive, and probably more extensive than those of Snu114p and Brr2p (Dix et al., 1998). Therefore, even though the interaction between Prp8p and this region of the U5 snRNA is being disrupted, Prp8p may still be associated with several other regions of U5 snRNA. With all deletion in the 3' side of U5 snRNA IL1 having such a large effect on associations of Brr2p, Snu114p and Prp8p, it is possible that this region of the U5 snRNA acts a protein docking site within the U5 snRNP, tethering Brr2p, Snu114p and Prp8p to the U5 snRNA. However, it should be considered that even a single nucleotide deletion in this small region of U5 snRNA may have a large effect on the secondary structure of U5 snRNA and may prevent, or severely impede, associations of Brr2p, Snu114p and Prp8p with U5 snRNA.

Data presented here illustrates that U5 snRNA Loop 1 and both sides of U5 snRNA IL1 are important for Brr2p, Snu114p and Prp8p associating with the U5 snRNA. It would appear that the sequence of the 5' side of IL1, not just the size of the loop, is important for protein association.

Even a single nucleotide deletion in the 3' side of the U5 snRNA IL1 had drastic effects on associations of Brr2p, Snu114p and Prp8p with the U5 snRNA. Genetic interactions of the N-termini of both Snu114p and Brr2p, and also the C-terminus of Snu114p with the 3' side of IL1 (Frazer et al., 2009). These data have led to the proposition that the 3' side of the U5 snRNA IL1 may act as a 'protein docking' site within the U5 snRNP. This is also supported by previous data demonstrating that Prp8p crosslinks to this position C112 in the 3' side of IL1. This would suggest a model in which the N- and C-termini of Snu114p, and the N-terminus of Brr2p and a region of Prp8p interact with the 3'side of IL1 of the U5 snRNA, tethering them to a common point in the U5 snRNP. To rule out the possibility that the effects on protein association are due to large changes in the structure of the U5 snRNA, chemical probing could be carried out to assess how much the U5 snRNA structure is changed by deletions in the 3' side of IL1. Also these immunoprecipitation experiments could be repeated using U5 snRNA with a sequence substitution of the 3' side of IL1. This may affect the structure of the U5 snRNA less than deletions, and would determine if the sequence of the 3' side of IL1 is important for protein associations with the U5 snRNA.

To summerise, data shown here demonstrates that the 3' side of U5 snRNA IL1 is important for the association of Brr2p, Snu114p and Prp8p (Figure 4.24). Data also suggests that Brr2p contacts the U5 snRNA down the 3' side of the U5 snRNA snd Snu114p contacts

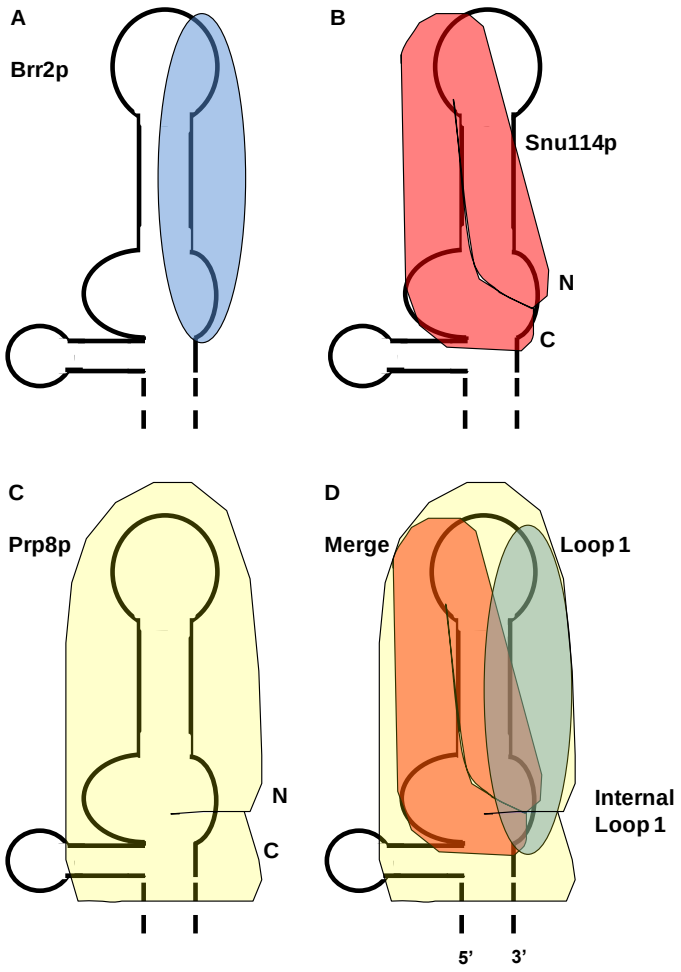

Figure 4.24 Proposed model of the association of Brr2p, Snu114p and Prp8p with Loop 1, stem 1 and Internal Loop 1 of the U5 snRNA. A. The 3' side of IL1 and the 3' side of Loop 1 of the U5 snRNA appear to be important for the association of Brr2p with the U5 snRNA, suggesting that Brr2p mainly associated with the 3' side of the U5 snRNA. B. Both sides of the IL1 and the 5' side of Loop 1 of the U5 snRNA are particularly important for the association of Snu114p with the U5 snRNA. Therefore it is thought that Snu114p interacts with IL1 and the 5' side of Loop 1 of the U5 snRNA. Furthermore, both the N- and C-termini of Snu114p have been shown to be involved in the interaction with the 3' side of IL1. C. Prp8p is known to interact directly with Loop 1 and both side of IL1 of the U5 snRNA. As the N- and C-termini of Prp8p are thought to interact with each other, and the C-terminus of Prp8p interacts with the C-terminus of Snu114p, it is predicted that the N- and C-termini of Prp8p will be situated at or near the 3' side of IL1 of U5 snRNA. D. Figure shows a merge of the predicted interactions of Brr2p, Snu114p and Prp8p with the U5 snRNA. This highlights the 3' side of IL1 of the U5 snRNA as a major site of protein interaction within the U5 snRNP.

the U5 snRNA mainly down the 5' side of the secondary strcrure of the U5 snRNA (Figure 4.24). The N- and C-termini of Snu114p are known to be involved in the interaction between Snu114p and the 3' side of U5 snRNA IL1 (This study and Frazer et al., 2009). Prp8p is known to interact directly with Loop 1 and both sides of IL1 (Dix et al., 1998). As the C-terminus of Prp8p is thought to interact with the C-terminus of Snu114p, and the N- and C-termini of Prp8p appear to interact with each other, it is predicted that the N- and C-termini of Prp8p are both present around the 3' side of U5 snRNA IL1 (Liu et al., 2006). This suggests some spatial organisation of Brr2p, Snu114p and Prp8p witin the U5 snRNP (Figure 4.24).

The fact that each U5 snRNA mutation does not affect Brr2p, Snu114p and Prp8p associations with U5 snRNA in the same way suggests that, even though there are extensive interactions between these three proteins, Brr2p, Snu114 and Prp8p do not just bind U5 snRNA as a protein complex. The requirements for the association of Brr2p, Snu114p and Prp8p with the U5 snRNA are slightly different for each protein.

However, data presented here does suggest that the association of Brr2p with U5 snRNA is effected by associations of Prp8p with U5 snRNA. Prp8p and Brr2p both showed more U5 snRNA association with deletion or substitution of the 5' side of U5 snRNA IL1 and less U5 snRNA association with Loop 1 deletions, compared to Snu114p association with the same mutants. As Ppr8p interactions with the U5 snRNA are so extensive, and no direct link between Brr2p and U5 has yet been found, it is probable that Brr2p interacts with U5 snRNA, at least partially, via Prp8p (Dix et al., 1998).

Previous work has suggested that Prp8p is required for the association of Snu114p with the U5 snRNA (Brenner and Guthrie, 2006). However, this study has demonstrated that Snu114p is able to associate with a U5 snRNA mutant that Prp8p is not able to associate with.

This would suggest that the role of Prp8p in the recruitment of Snu114p into the U5 snRNP, is not to enable Snu114p to interact with U5 snRNA by indirect interactions with Prp8p.

This study has demonstrated that the association of Brr2p with U5 snRNA is affected by Prp8p, and are possibly indirect via Prp8p, and that Snu114p can associate with the U5 snRNA, without Prp8p associating with the U5 snRNA. These observations were surprising, as localisation studies of Brr2p, Snu114p and Prp8p within the tri-snRNP have shown that Snu114p and Prp8p are localised to similar regions of the U5 snRNP within the tri-snRNP and Brr2p is localised to another region of tri-snRNP (Hacker et al., 2008). This would indicate that Snu114p and Prp8p may be binding the U5 snRNA as a complex. Data presented here suggests that it is more likely that Brr2p and Prp8p are binding as a complex. However, the localisation studies of Hacker et al were carried out using C-terminally tagged proteins and the location of the tag was detected. So these studies tell us the localisation of the C-termini of Brr2p, Snu114p and Prp8p. In support of the localisation data, it has been shown that the C-terminus of Snu114p interacts with the C-terminus of Prp8p (van Nues and Beggs, 2001). Work here indicates that the associations of Brr2p with the U5 snRNA may partially be indirect via Prp8p. The fact that the N-terminus of Brr2p has been shown to interact with the C-terminus of Prp8p, and that Brr2p also interacts with the N-terminus of Prp8p, supports this theory (Liu et al., 2006; van Nues and Beggs, 2001). It would be interesting for the Hacker et al localisation studies to be expended and carry out further localisation studies using antibodies to different regions of Brr2p, Snu114p and Prp8p, to determine where other protein regions are localised, and if any other protein regions co-localise.

As mentioned the 3’ side of the U5 snRNA IL1 is important for the association of the U5 snRNA. This study has also demonstrated the importance of the 5’ side of the U5 snRNA IL1

in the association of Snu114p with the U5 snRNAs. Deletion of the 5' side of U5 snRNA IL1 abolished the association of Snu114p, but not Prp8p and Brr2p. This demonstrated that protein-protein interactions between Snu114p and Prp8p are not sufficient for Snu114p association with the U5 snRNA and ability of Snu114p to associate to some extent with the U5 snRNA with out Prp8p being associated with U5 snRNA. These data suggests that association of Snu114p with the U5 snRNA requires a direct interaction with the 5' side of U5 snRNA IL1. This is supported by the identification of a crosslink between Snu114p and the 5' side of the U5 snRNA IL1 (Dix et al., 1998). The importance of both sides of IL1 in association of Snu114p is also supported by genetic interactions between the N- and C-termini of Snu114 and the 3' side of the U5 snRNA IL1 (Dix et al., 1998; Frazer et al., 2009) (This study). These data indicate that the U5 snRNA IL1 is the major site of interaction between Snu114p and U5 snRNA.

The suggestion that the U5 snRNA IL1 is the major site of Snu114p interaction with the U5 snRNA supports a suggested model in which Snu114p positions U5 Loop 1 into a pocket of Prp8p where the interactions with the pre-mRNA substrate occurs (Dix et al., 1998; Staley and Guthrie, 1998). IL1 along with Stem 1, of the U5 snRNA are situated below Loop 1 in the secondary structure of the U5 snRNA, and changes in IL1 and Stem 1 would most likely alter the position of Loop 1 (Frank et al., 1994). Interactions of Snu114p with the IL1 of the U5 snRNA, would suggest that conformational changes within Snu114p would alter the position of Loop 1 within the spliceosome, probably between the two steps of splicing (Dix et al., 1998; Staley and Guthrie, 1998). The repositioning would occur in a similar manner to the actions of EF-2 during translocation of tRNA from the A to P site within the ribosome, a step which involves conformational changes of EF-2 triggered by GTP hydrolysis. This would position U5 Loop 1 to the active site of the spliceosome, facilitating U5 binding to the exons

and the two catalytic steps of splicing (Abelson, 2008; Dix et al., 1998; Staley and Guthrie, 1998).

Studies using U5 snRNA mutants containing deletions within the conserved Loop 1 suggest that of all the nucleotides of the U5 Loop 1, nucleotides of the 5' side of the loop are the most important for associations of Snu114p, and nucleotides of the 3' side are most important for associations of Brr2p. This reveals a spatial organisation of protein association with U5 Loop 1, with Snu114 associating with the 5' side and Brr2p associating with the 3' side. In contrast the size of Loop 1, rather than specific nucleotides, is important for associations of Prp8p with the U5 snRNA. This further supports the model mentioned earlier, in which Snu114p acts to position U5 Loop 1 into a pocket within Prp8p at the active site of the spliceosome, facilitating U5 binding to the exons and the two catalytic steps of splicing (Abelson, 2008; Dix et al., 1998; Staley and Guthrie, 1998).

It should be noted that interactions being investigated here may not be direct, and given the intimate protein-protein interactions that exist between Brr2p, Snu114p and Prp8p, it is likely that some of these interactions with U5 snRNA will be indirect (Liu et al., 2006). It should also be considered that the association of other U5 snRNP or tri-snRNP proteins may also be effected by the mutations made in the U5 snRNA, such as Prp6p which interacts with Brr2p, Snu114p and Prp8p (Liu et al., 2006). So, interactions of Brr2p, Snu114p and Prp8p with the U5 snRNA, disrupted by mutations made in U5 snRNA, may also be due to the disruption of the interaction between other proteins and the U5 snRNA.

4.2.6 Genetic interactions of *BRR2* and U5 snRNA

To further define the vital interactions between Brr2p and the U5 snRNA required for U5 snRNP and U4/U6.U5 tri-snRNP formation, and function of Brr2p at the catalytic core of the spliceosome, a genetic screen was carried out with *BRR2* and U5 snRNA mutants. Two new lethal *brr2* mutants have been identified and the genetic screen revealed novel interactions between the N-terminus and first helicase domain of Brr2p and the 3' side of U5 snRNA IL1. This implicates the N-terminal portion of Brr2p in interactions with the U5 snRNA.

Of the four novel *BRR2* mutants constructed and tested in this study, Brr2-P841L and Brr2-G873L were both lethal. These mutants contain amino acid substitutions in the first helicase domain. This region contains the DExH box protein consensus sequences required for ATPase activity. Brr2-G873L contains a mutation that changes the first residue in one of these consensus sequences (GRAGR), so the lethal phenotype may well be due to a disruption of the ATPase activity of Brr2p (Lauber et al., 1996) The mutation present in Brr2-P841L does not lie within one of the DExD box consensus sequences, but due to its close proximity to these consensus sequences and the lethal phenotype, it is possible that this amino acid 841 of Brr2p has direct some role in ATP binding or hydrolysis, or its substitution effects protein regions involved in ATPase activity of Brr2p. Defects in ATPase activity with either mutation could be tested in ATPase assays containing radioactive ATP, and measuring the amount of ATP left in assay supernatant, determining how much has been used, after the addition of purified mutant or wild-type Brr2p (Schneider et al., 2002). Amino acids P841 and G873 of Brr2p lie either side of a putative β-hairpin region within the first Hel308 domain. This β-hairpin is thought to be involved in disruption of double stranded DNA and disruption of the β-hairpin has been shown to cause a growth defect (Zhang et al., 2009). Therefore in the case of Brr2-

P841L and -G873L, the lethal phenotype may be due to disruption of the β-hairpin and the helicase activities of Brr2p.

The other two novel *brr2* mutants, Brr2-R295I and Brr2-F1149I, were both viable at all temperatures tested, indicating that these mutations, located in the N-terminal domain and first Sec63 domain of Brr2p respectively, do not significantly disrupt the function of Brr2p. The growth test of Brr2-E610G revealed that this mutation caused a cold sensitive phenotype, with cells containing this mutation failing to grow at 16°C. This cold sensitive phenotype of Brr2-E610G has also been shown in a previous study (Raghunathan and Guthrie, 1998). The previously published Brr2-E909K mutation has been shown to be temperature sensitive above 33°C (which is supported by Brr2-E909K being lethal at 37°C when tested in this study), and has a slow growth phenotype at temperatures between 23°C and 30°C (Xu et al., 1996). When tested in this study Brr2-E909K was viable at 16°C, RT and 30°C. The difference in results seen here may be due to the different methods of testing for viability. In the previous study viability was tested by streaking colonies onto 5FOA containing plates, but in this study spot plating of serial dilutions onto 5FOA containing plates was carried out, which is more accurate. When streaking colonies onto plates, the number of cells being streaked out is not measured, but when carrying out spot plating, cells are grown in culture and a defined amount of culture of a specific OD is spotted onto each plate. Brr2-N1104L was shown to be viable at all temperatures tested in this study. This mutation is the yeast equivalent of a mutation that is linked to Retinitis Pigmentosa in humans, and has been shown to cause a defect in U4/U6 unwinding in yeast, but shows no temperature sensitivity at 15°C (Zhao et al., 2009). Brr2-R1107L is also the yeast equivalent of a mutation that is linked to Retinitis Pigmentosa in humans (Zhao et al., 2009). Previous study of Brr2-R1107L has shown this mutation causes a defect in U4/U6 unwinding, that is worse than the unwinding defect seen in Brr2-N1104L, and

a temperature sensitive phenotype at 15°C (Zhao et al., 2009). Temperature sensitive testing of both mutants here has demonstrated a growth defect at low temperatures (16°C) and 37°C for Brr2-R1107L, but no growth defect of N1104L at any temperature tested. The difference in U4/U6 unwinding defects and temperature sensitivity between these mutants may be due to the fact that amino acid R1107 is conserved between yeast and humans, whereas the human equivalent to the yeast amino acid N1104, is actually a serine, indicating that amino acid R1107 is more conserved and may be more important in U4/U6 snRNA unwinding, than N1104. Brr2-R1107A, another yeast equivalent of a human Retinitis Pigmentosa causing mutation, was also shown in a previous study to be cold sensitive (Small et al., 2006). Brr2-R1107A was shown here, to be cold sensitive at 16°C and 37°C, like Brr2-R1107L. This would suggest it is the lack of the arginine residue, rather than the presence of the alanine or leucine residue, that is responsible for the growth defect. The two *brr2* mutants containing amino acid substitutions altering ATPase motifs in the second helicase like domain, Brr2-G1375D,K1376N and Brr2-D1474G, were both viable at all temperatures tested. It has already been shown that these mutants are viable, and this study showed, they were viable at all temperatures tested (Kim and Rossi, 1999). This supports the observation that only the first helicase-like domain of Brr2p is required for cell viability and splicing (Kim and Rossi, 1999). All mutations constructed in the C-terminal half of Brr2p in this study, were viable at all temperatures. This suggests that the conserved sequence motifs present in the C-terminal half of Brr2p are not required, and that the general shape of the C-terminal half of the protein, rather than specific sequence motifs, is important for the function of Brr2p. To test if this is a general trend, more mutations in the C-terminal half of the protein should be made, particularly in the second Sec63 domain (S2).

A genetic screen was carried out to test for synthetic lethal interactions between these *brr2* mutants and a set of U5 snRNA mutants. A U5 mutant containing a C111 deletion was used in this screen. This deletion has been used in a different yeast strain in this study, in which it was lethal at 30°C and 37°C, and sick at 16°C and RT. However, when present in the yeast strain used in this screen, it was viable at 16°C, RT and 30°C, and sick at 37°C. This must be due to differences in yeast strain genetic background. The screen detected genetic interactions between several *brr2* mutants and the 3' side of U5 snRNA IL1. Brr2-R295I and Brr2-E610G were both lethal at RT with U5-ΔC112,G113. Brr2-R1107A and Brr2-R1107L were lethal with U5-ΔC112,G113 at 30°C. This suggests a role for the N-terminal region, the first helicase-like domain and first Sec63 domain of Brr2p in interactions with the 3' side of U5 snRNA IL1. This is supported by the immunoprecipitation experiments showing that deletions in the 3' side of U5 snRNA IL1 effect the association of U5 snRNA with Brr2p. Genetic interactions have also been identified between the N-terminus and C-terminal half of Snu114p and the 3' side of U5 snRNA IL1 (Figure 3.21) (Frazer et al., 2009). This region of Snu114p in humans has been shown to interact with Brr2p (Liu et al., 2006). It is, therefore, possible that Brr2p associates with U5 snRNA in the 3' side of IL1, through interactions with Snu114p. No synthetic lethal interactions were found between *brr2* mutants and U5 snRNA containing mutations in Loop 1 or the 5' side of IL1. This supports the idea that Brr2p may associate with these regions of U5 snRNA partially through Prp8p. Genetic studies similar to this have been carried out with Snu114p (Frazer et al., 2009) (this study). Genetic interactions between Prp8p and the U4 and U6 snRNAs have been discovered (Kuhn and Brow, 2000; Kuhn et al., 2002), but to investigate interactions between Prp8p and the other snRNAs, additional genetic screening could be carried out.

Following on from the experiments described here, several paths of investigation could be taken to increase our knowledge of the protein-RNA interactions present within the U5 snRNA and U4/U6.U5 tri-snRNP. To determine which interactions of Brr2p, Snu114p and Prp8p with the snRNAs are direct, crosslinking could be carried out prior to immunoprecipitation and primer extension. To define the regions of U4 and U6 snRNA that Brr2p, Snu114p and Prp8p interact with in the tri-snRNP, immunoprecipitations could be carried out, similar to those performed in this study, using U4 and U6 snRNA mutants. Immunoprecipitation experiments or EMSA using purified fragments of Brr2p, Snu114p and Prp8p could be carried out to determine which regions of each protein are involved in snRNA interactions. To increase our understanding of the interactions of Prp8p with snRNAs, and the regions of Prp8p involved in these interactions, a genetic screen using Prp8p and snRNA mutants could be carried out. Further localisation studies could also be carried out, using antibodies to different regions of, or at least the N-termini of, Brr2p, Snu114p and Prp8p, so we know where more than just the C-termini of each protein is localised with in the tri-snRNP. All of these experiments will help to elucidate the complex web of protein-RNA interactions present within the U5 snRNP and the tri-snRNP, and define the regions of protein involved in snRNA interactions, and how the protein-protein or protein-RNA interactions change throughout splicing. Increasing our understanding of where proteins are situated within the U5 snRNP and tri-snRNP will help to define the functions the U5 snRNP proteins and how these proteins communicate together to control and carryout essential processes throughout pre-mRNA splicing.

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